New Biodegradable Peptide-based Polymer Constructs

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PhD thesis with summary in Dutch
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New Biodegradable Peptide-based Polymer Constructs

Nieuwe biodegradeerbare polymeerconstructen gebaseerd op peptiden

(met een samenvatting in het Nederlands)

Proefschrift

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Chapter 1

General Introduction

1.1 Peptide-based polymers

There is a great interest in peptide-based biopolymers, since they can be applied for a variety of purposes such as drug delivery devices, scaffolds for tissue engineering and -repair, and as novel biomaterials. Peptide-based polymers are common in Nature and often exhibit special characteristics. Spider silk for example is a remarkably strong material. Although, its tensile strength is comparable to that of high-grade steel, its density is substantially lower.[1,2] Peptide-based polymers are also found inside the human body. Collagen for example, is the most abundant protein in mammals and the major component of the extracellular matrix that supports most tissues and gives cells mechanical strength.[3] Elastin allows many tissues in the body to resume their shape after stretching or contracting.[4] Peptide-based polymers are especially interesting because their primary structure allows the specific cleavage by endogenous proteases,[5] thereby reducing the risk of accumulation and toxic side-effects.[6] This specific cleavage by endogenous proteases can also be used for site-specific release of drugs.[7] Since it is very difficult and expensive to harvest these peptide-based biopolymers in large quantities from Nature, and the desire to introduce modifications to modulate their properties, necessitate the development of synthetic methods to obtain such peptide-based polymers.

1.2 Current approaches to synthesize peptide-based polymers

Currently, two different approaches for the synthesis of such peptide-based polymers are in use. In the first approach, peptides or amino acids are N-terminally coupled onto a polymerizable group. The amino acid-based monomers are polymerized via radical, anionic, and cationic polymerization, which yield polymers with amino acid and peptide moieties in the side chains. (Figure 1 (1)).[8,9] In the second approach, peptide-based polymers consist of peptides in the backbone (Figure 1 (2)).[10] This approach implies that at several positions of the peptide polymer backbone, the native amide bond is replaced by a suitable amino bond isostere, *e.g.* a triazole moiety (*vide infra*).

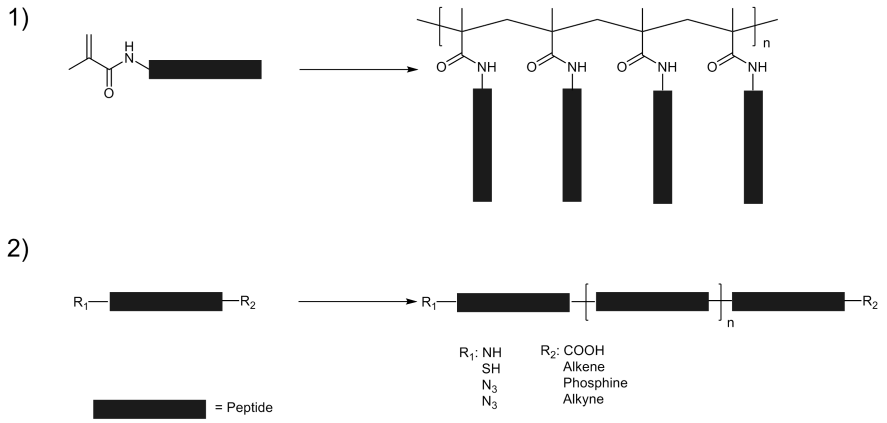


Figure 1: Two approaches to synthesize peptide-based polymers: polymers with amino acid moieties grafted onto the side chain (1) and synthesis of polymers with amino acid moieties in the backbone (2).

1.2.1 Synthesis of polymers with amino acid moieties grafted onto the side chain

Various examples of α-amino acid-based methacrylamide polymers have been reported in literature. For example, Handa and coworkers[11] polymerized an L-leucine-based methacrylamide (*N*-methacryloyl-L-leucine methyl ester) via radical polymerization to obtain high molecular weight polymers (Figure 2). The synthesized polymers formed highly ordered structures that might be used as biocompatible materials such as artificial skins and fibers.

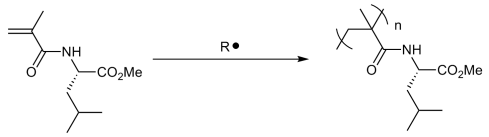


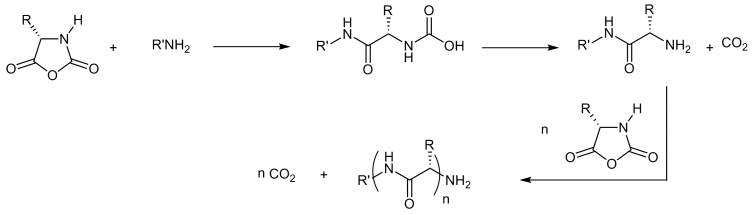
Figure 2: Radical polymerization of *N*-methacryloyl-L-leucine methyl ester.[11]

Endo and coworkers[12] synthesized two acrylamides containing proline and hydroxyproline moieties. The acrylamides were polymerized by reversible addition fragmentation chain transfer (RAFT) polymerization to yield well-defined amino acid based polymers with thermoresponsive properties. Thermosensitive polymers are characterised by a so-called cloud point (CP) or lower critical solution temperature (LCST). Thermoresponsive polymers are soluble in water below the LCST or CP and become insoluble when the temperature is raised above the LCST. By co-polymerizing the two monomers in various ratios the cloudpoint (CP) of the polymers could be tailored in the range of 17-50 °C. Thermoresponsive polymers with a CP around human body temperature are under investigation for biomedical and pharmaceutical applications (chapter 7).[13,14]

However, this class of polymers, with amino acid moieties in the side chains, often have a synthetic backbone with a rather low biodegradability, which might lead to cytotoxicty, especially when the molecular weight of the polymer is above the renal threshold value (typically 30 kDa).[15,16] Furthermore, some peptide-based biopolymers derive their structural-, mechanical- and biological properties from their backbone sequential repetitions; these properties might be lost when the peptides are N-terminally grafted onto a synthetic polymer backbone.

1.2.2 Synthesis of polymers with amino acid moieties in the backbone

Conventional solid-phase peptide synthesis is not useful or practical for the synthesis of large peptide-based polymers (> 100 amino acid residues). Also conventional solution-phase peptide synthesis would be far too labor-intensive and expensive. An economical and expedient process for the synthesis of relatively high molecular weight polypeptides is the polymerization of α-amino acid-N-carboxyanhydrides (NCAs) (Scheme 1).[17-24]



Scheme 1: Synthesis of peptide-based polymers via NCA polymerization.

NCA polymerization has primarily been used to synthesize polymers consisting of a single amino acid residue, like poly-Glu or poly-Lys. Furthermore, NCA polymerization requires that functional groups in the side chain of the amino acids are protected to avoid side-reactions. The ring-opening polymerization of protected amino acid N-carboxyanhydrides is frequently used for the synthesis of co-polymers with synthetic and polypeptide block segments. Schlaad and coworkers[24] for example used a combination of cationic/anionic- and NCA polymerization reactions for the synthesis of poly(2-isopropyl-2-oxazoline)-β-poly(L-glutamate). This co-polymer consists of a thermosensitive block (poly(2-isopropyl-2-oxazoline) (LCST of 40 °C) and a pH-sensitive block (poly(L-glutamate)). The combination of thermo- and pH responsiveness can be used for the triggered release of drugs.
Other frequently used methods are the use of activated peptide esters[25] and the application of condensing agents such as diphenylphosphorylazide,[26,27] carbodiimides and acid chlorides.[28] These methods suffer from the same drawbacks. In the ideal situation the polymerization reaction should be performed with unprotected amino acids, in high yield under mild conditions.

1.3 The 'click' concept

In 2001, Sharpless and co-workers coined the concept of "click chemistry" to classify a particular set of nearly perfect reactions, *vide infra*.[29] By means of "click reactions", large (bio)macromolecules can be synthesized by coupling small building blocks via heteroatom-containing linkages. Such a coupling reaction should meet several criteria: it should be modular, high yielding, generate only harmless side-products, and it should be carried out under mild reaction conditions, preferentially in the presence of other functional groups using readily available starting materials and reagents.
There are several well-known reactions that comply with the "click chemistry" concept, with among others, the thiol-ene coupling,[30] the Staudinger ligation,[31-33] native chemical ligation,[34-36] the amidation reaction between thio acids and sulfonyl azides (sulfo-click)[37-42] and the copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC).[43-45] In the next section several click reactions are briefly summarized; for more information the most imported reviews are listed in the references.

1.3.1 Thiol-ene coupling

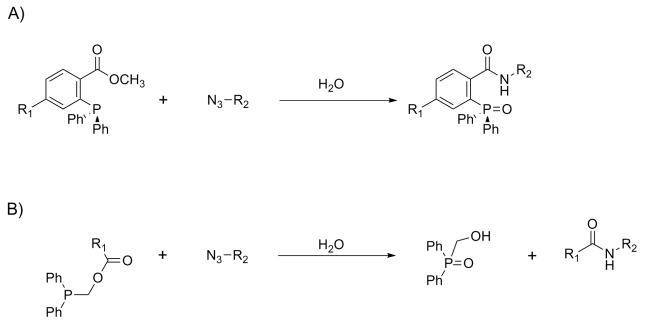
The century-old reaction between thiols and alkenes, called the thiol–ene coupling (TEC) has recently regained new interest as an attractive ligation method. In this Michael-type reaction a thiol reacts with various (activated) alkenes such as acrylates, acrylamides, vinyl sulfones or maleimides to form thioethers (Scheme 2).[46,47] The thiol-ene coupling is compatible with water and the formed thioether linkage is stable in a wide range of chemical environments, such as strong acid and basic media as well as oxidizing and reducing conditions.

Scheme 2: The thiol-ene coupling reaction.

The TEC have been frequently used for the synthesis of hydrogels (chapter 2). For example, Hubbell and coworkers [48,49] synthesized hydrolytically degradable PEG hydrogels via TEC. They used a four-arm and an eight-arm PEG spacer functionalized with acrylate end-groups and either dithiothreitol or PEG-dithiol as cross-linking agents. The PEG-acrylate contains an ester bond that can be cleaved by chemical hydrolysis. By altering the molecular weight of the PEG derivative, or by varying the concentration of either the cross-linker or the PEG-acrylate molecules, hydrogels with different swelling ratios were obtained. The different swelling ratios also resulted in different degradation kinetics.

1.3.2 The Staudinger ligation

The Staudinger ligation is a modification of the classical Staudinger reaction and has been developed by Bertozzi and coworkers in 2000 to modify the surface of cells.[33] In the Staudinger ligation a phosphine reacts with an azide in the presence of water (Scheme 3). The reaction occurs with such a high selectivity and high speed, that the reaction can be applied toward complex biomolecules in living cells. However, in the classical Staudinger ligation, a non-native group is incorporated at the ligation site (Scheme 3A). Therefore Bertozzi and cowokers[31] developed a "traceless" Staudinger ligation, resulting into a native amide bond at the ligation site (Scheme 3B).



Scheme 3: The "classical" Staudinger ligation (A) and the "traceless" Staudinger ligation.

The "traceless" Staudinger ligation is an excellent method for the selective formation of an amide bond in the presence of other functional groups. However, low yields and moderate reaction rates make these chemoselective reactions unsuitable for polymer synthesis.

1.3.3 Native chemical ligation

Native chemical ligation (NCL) of unprotected peptide segments was developed by Kent and co-workers in 1994 based on the initial ideas of Theodor Wieland.[34] In native chemical ligation a peptide containing a C-terminal thioester reacts with another peptide containing an N-terminal cysteine to form an amide bond (Figure 3).

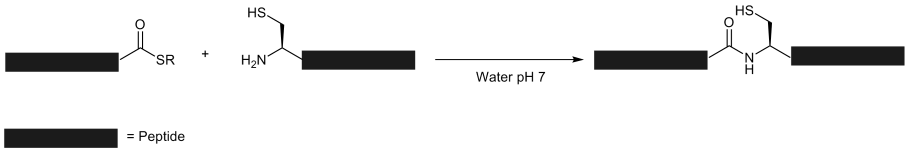
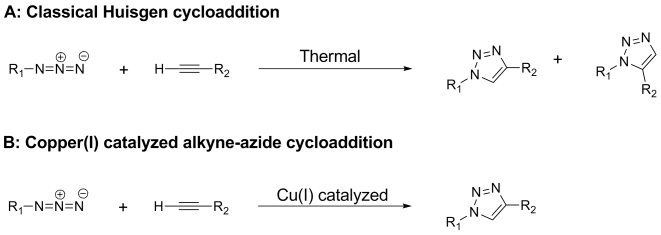


Figure 3: Native chemical ligation.

The reaction takes places in aqueous solution, in the presence of guanidine·HCl at neutral pH and gives near-quantitative yields. The strength of NCL is that the reaction takes place with unprotected peptides as starting material and after the reaction a native peptide bond is formed. A drawback of the NCL is the need for a cysteine residue at the ligation site(s).

1.3.4 The copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC)

In the CuAAC reaction, an organic azide reacts with a terminal alkyne to form a triazole ring, similar to the classical Huisgen cycloaddition reaction (Scheme 4A).[50] In 2002, Meldal and co-workers as well as Sharpless and co-workers independently discovered that in the presence of a copper(I) catalyst, the 1,3-dipolar cycloaddition between an azide and alkyne proceeds with an increase reaction rate, even at ambient reaction conditions.[43-45] Moreover, with the classical Huisgen cycloaddition reaction a mixture of the 1,4- and 1,5-substituted triazole derivatives is obtained.[51], while with the copper(I) catalyzed version only the 1,4-substituted triazole is formed (Scheme 4B).



Scheme 4: The classical Huisgen cycloaddition and the copper(I) catalyzed alkyne-azide cycloaddition.

Recent studies showed that the 1,2,3-triazole moiety, which is formed during the click reaction, has clear similarities to the native peptide amide bond in terms of distance and planarity. Therefore, it is an effective mimic of a peptide amide bond and has been used as a dipeptide replacement in β-strands and α-helical coiled coils.[52,53] The applicability of the chemoselective azide-alkyne coupling in the presence of other (unprotected) functional groups and the topological similarities between the native

peptide amide bond and a 1,2,3-triazole moiety was the rationale to explore the 1,3-dipolar cycloaddition reaction for the synthesis of peptide-based polymers.

1.4 Aim and outline of this thesis

The aim of this thesis is to develop new methodologies for the synthesis of peptide-based polymers for pharmaceutical and biomedical applications. In **chapter 2**[54] an overview is given about the synthesis and application of biomedical and pharmaceutical polymers synthesized via two click reactions studied frequently in recent literature, namely the copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) and the thiol-ene coupling reaction.

In **chapter 3**[55] a model dipeptide azido-phenylalanyl-alanyl-propargyl amide is polymerized by a microwave-assisted copper(I)-catalyzed alkyne-azide cycloaddition. Depending on the reaction conditions, it was found that the click reaction could be directed either to large linear polymers (up to 300 amino acid residues) or toward medium-sized peptide macrocycles (4 to 20 amino acid residues). As a proof of principle, a second monomer consisting of N_3-βAla-Pro-Gly-Ser-propargyl amide, representing the repetitive amino acid sequence within spider silk, was synthesized and polymerized.

In **chapter 4**[56] the microwave-assisted copper(I)-catalyzed 1,3-dipolar cycloaddition reaction was used to synthesize peptide triazole-based polymers from two novel biodegradable monomers: azido-phenylalanyl-alanyl-lysyl-propargyl amide and azido-phenylalanyl-alanyl-glycoloyl-lysyl-propargyl amide. The selected monomers have sites for enzymatic degradation as well as for chemical hydrolysis. It was demonstrated that the synthesized polymers were sensitive for both enzymatic and chemical hydrolysis.

In **chapter 5** the copper(I)-catalyzed 1,3-dipolar cycloaddition reaction was used to synthesize enzymatically degradable PEG-based hydrogels, based on alkyne-functionalized star-shaped PEG derivatives and a protease sensitive peptide sequence functionalized with two azide moieties. It was shown that the rheological properties of the hydrogels could be tailored by varying the reaction conditions and that the hydrogels could be degraded by trypsin.

In **chapter 6**[57] the microwave-assisted copper(I)-catalyzed 1,3-dipolar cycloaddition reaction was used for the synthesis of cyclic oligomers based on the amyloidogenic Aβ(16-22) peptide sequence. These cyclic oligomers, on their turn, were used as building blocks in a self-assembly process to obtain supramolecular polymers. Properties and characteristics of these oligomers and supramolecular polymers were studied with transmission electron microscopy, FTIR and CD.

In **chapter 7** LCST (lower critical solution temperature) polymers based on N^{α}-methacryloyl alkyl esters of serine and threonine, were synthesized by free radical polymerization. LCST polymers are soluble in water below the LCST and become insoluble when the temperature is raised above the

LCST. It was shown that the LCST of the polymers could be tailored by varying the type of the ester moiety as well as the amino acid derivative. The esters could be degraded by chemical hydrolysis, which resulted in an increase in LCST of the polymer. By synthesizing a block-co-polymer with PEG, thermoresponsive particles were created. Upon hydrolysis of the ester bonds these particles destabilized.

Chapter 8 gives a brief summary of the thesis. With a special focus on the possibilities to synthesize peptide-based polymers via the copper(I)-catalyzed 1,3-dipolar cycloaddition reaction, the future perspectives of such polymer constructs for pharmaceutical applications are outlined.

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Chapter 2

Synthesis and Applications of Biomedical and Pharmaceutical Polymers via Click Chemistry Methodologies

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Bioconjugate chemistry (accepted)

2.1 Abstract

In this chapter the synthesis and application of biomedical and pharmaceutical polymers synthesized via the copper(I)-catalyzed alkyne-azide cycloaddition, the thiol-ene reaction or a combination of both click reactions are reviewed and discussed. Since the introduction of both "click" methods, numerous articles have disclosed new approaches for the synthesis of polymers with different architectures –e.g. block- and graft-copolymers, dendrimers, and hydrogels– for pharmaceutical and biomedical applications. By describing selected examples, this chapter gives an overview of the possibilities and limitations that these two "click" methods may offer.

2.2 Introduction

The use of polymers for biomedical and pharmaceutical applications has gained an enormous impact during the past decades. Polymers can be applied in drug delivery systems, scaffolds for tissue-engineering and -repair, and as novel biomaterials.[1]. These applications have led to an increasing demand of well-defined polymers with tailorable properties. The emergence of the "Click Chemistry" concept greatly facilitated the synthesis of these polymers. Click chemistry provides very attractive possibilities for (bio)conjugation reactions because it can be performed at ambient conditions with readily available starting materials. In recent years, click chemistry has also been applied for the synthesis of polymers with different architectures (including block- and graft co-polymers) including polymers with pharmaceutical and biomedical applications. This review will focus on the different click chemistry strategies to synthesize this class of polymers and will describe some selected examples in more detail to highlight their applications in the biomedical and pharmaceutical field.

In 2001, Sharpless and co-workers coined the concept of "click chemistry" [2] to classify a particular set of nearly perfect reactions among others the Cu(I)-catalyzed 1,3-dipolar cycloaddition, *vide infra*. By means of the "click reaction" concept, large (bio)macromolecules can be synthesized by coupling small building blocks via heteroatom containing linkages. Such a coupling reaction should meet several criteria: it should be modular, high yielding, generate only harmless side-products, and it should be carried out under mild reaction conditions, preferentially in the presence of many other functional groups using readily available starting materials and reagents.
There are several well-known reactions that comply with the "click chemistry" concept, including the hetero-Diels-Alder reaction,[3] the thiol-ene coupling,[4] the Staudinger ligation,[5-7] native chemical ligation,[8,9] the amidation reaction between thio acids and sulfonyl azides (sulfo-click) [10-15] and

presently the most popular and the already mentioned copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC).[16-18]

Since the large number of recent original publications and (specialized) reviews that have been published during the last years on CuAAC, (for a detailed list see Table 1), this chapter will be focused on the synthesis of polymers and/or their post-synthetic modification/functionalization by the CuAAC and the thiol-ene reaction. In addition, it will describe some selected examples of biomedically and pharmaceutically relevant polymers that have been synthesized by these two click reactions. (This chapter covers the literature until April, 2009).

Table 1: List of reviews dealing with the synthesis of large (multivalent) systems and macromolecules by the CuAAC.

An overview of polymer synthesis and modification via the CuAAC.	Binder et al.[19,20]
Review about click polymerization reactions as well as ligation and functionalization of polymers.	Meldal [21]
Reviews dealing with the CuAAC in material science.	Nandivada et al.[22], Lutz[23], and Johnson et al.[24]
Review about synthesis of peptidomimetics via CuAAC.	Angell et al.[25]
Overview of carbohydrates and peptide-based dendrimers/polymers.	Pieters et al.[26]
Reviews about the construction of biohybrid materials.	Dirks et al.[27] Le Droumaguet et al.[28] and Lutz[29]
Review with emphasis on the synthesis of well defined polymer architectures.	Fournier et al.[30]
Review about various cross-linking methods of micelles including CuAAC.	Read et al.[31]
An overview of the synthesis of biodegradable polyesters by ring-opening polymerization and CuAAC.	Lecomte et al.[32]
A review about copper-free azide-alkyne cycloaddition reactions.	Lutz [33]
Other important reviews dealing with CuAAC.	Meldal and Tornøe.[34], Bock et al.[35], Moses et al.[36], Moorhouse et al.[37]

2.3 General considerations

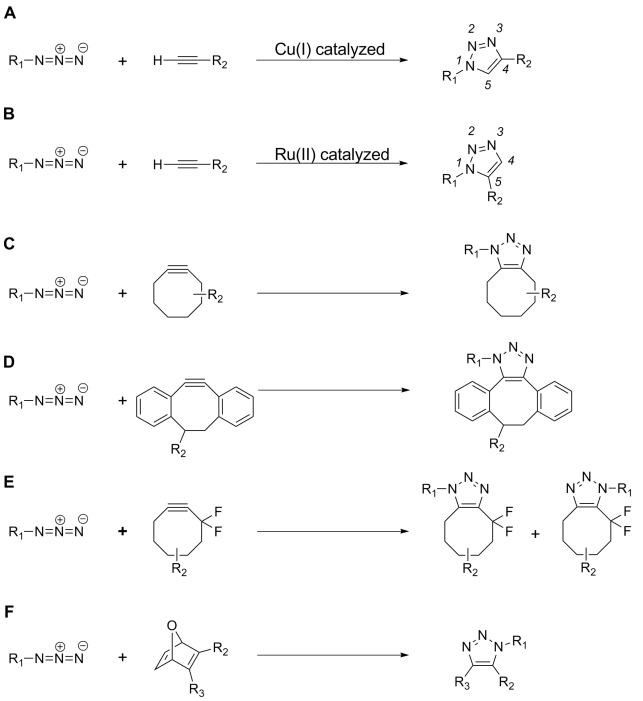
The Cu(I) catalysis of the well known 1,3-dipolar cycloaddition reaction (CuAAC) between an azide and an alkyne [38] was discovered in 2002 independently by the groups of Meldal[16,17] and Sharpless.[18]

In this cycloaddition reaction, an organic azide reacts with an alkyne to form a triazole ring, similar to the classical Huisgen cycloaddition reaction.[38] However, in the presence of copper(I) the reaction proceeds faster and even under ambient reaction conditions (Scheme 1A). Moreover, in the presence of copper(I) only the 1,4-disubstituted triazole ring is formed in contrast to the classical Huisgen cycloaddition reaction in which both 1,4- and 1,5-disubstituted triazole regioisomers are formed.[39] Since this Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction is very selective, it is highly compatible with almost all other functional groups present in biological macromolecules such as proteins, polysaccharides and DNA/RNA. Additionally, the CuAAC can be performed in aqueous media with a high reaction rate and generally leads to good product yields. Moreover, recent studies showed that the 1,2,3-triazole moiety, which is formed during the Cu(I)-catalyzed 1,3-dipolar cycloaddition, is similar to the (peptide)-amide bond in terms of geometry. Therefore, the triazole moiety has been suggested as a mimic of a peptide-amide bond and has been used for example as a dipeptide isostere in β-strands and α-helical coiled coils.[40-42]

A potential drawback of the Cu(I)-catalyzed cycloaddition reaction for the synthesis of polymers aimed for biomedical and pharmaceutical applications is the cytotoxicity of Cu(I). This can be especially troublesome when it is difficult to remove the copper catalyst from the synthesized polymers. Therefore, the use of ring strain has been investigated as an alternative for Cu(I) as catalyst to activate acetylenes. Wittig and Krebs [43] reported already in 1961 that the reaction between a cyclooctyne and phenyl azide resulted in the formation of a triazole functionality (Scheme 1C) and in 2004, Bertozzi and coworkers [44] demonstrated that the [3+2] cycloaddition of azides and cyclooctyne derivatives occurs under physiological conditions. However, the first generation of cyclooctynes was hampered with a relatively low reactivity towards azides, as compared to the CuAAC, resulting in long reaction times and lower coupling efficiencies. To improve their reactivity, Boons and coworkers [45] synthesized several 4-dibenzocyclooctynols (Scheme 1 D). By introducing aromatic moieties to the cyclooctyne, additional ring strain was created and also better conjugation was achieved as an additional factor to increase the reactivity of the alkyne. However, the first generation of these cyclooctynes and 4-dibenzocyclooctynols was rather insoluble in water, and in an attempt to improve the reactivity and water solubility, Bertozzi and coworkers designed and synthesized a second generation of difluorinated cyclooctynes [46,47] (Scheme 1E). These difluorinated cyclooctynes possess similar reaction kinetics as the Cu(I)-catalyzed cycloaddition reaction. Unfortunately, these difluorinated cyclooctynes are rather difficult to synthesize.[48]. A slightly different approach toward Cu-free click reactions was explored by Rutjes and coworkers.[49] They used a tandem [3+2] cycloaddition-retro-Diels-Alder ligation method in which trifluoromethyl-substituted oxanorbornadiene derivatives react with an azide to form a triazole linkage (Scheme 1F). This approach was successfully applied to labeling proteins and to the synthesis of pegylated oligopeptides

in various media at ambient temperature. Although the trifluoromethyl-substituted oxanorbornadiene derivatives are much easier to synthesize than difluorinated cyclooctynes, they have a lower reactivity toward azides compared to the difluorinated cyclooctynes. Recently, an overview of the development and perspective of copper-free azide–alkyne cycloadditions was given by Lutz.[33]

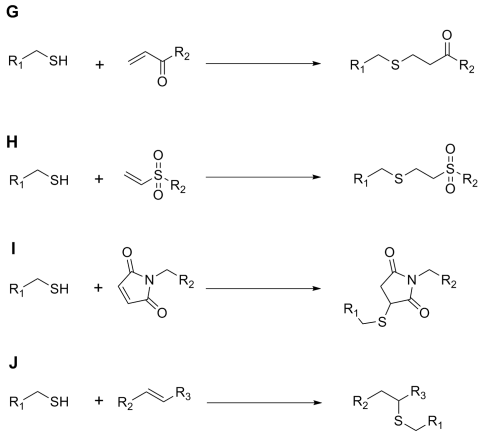
While the CuAAC generally yields a 1,4-disubstituted 1,2,3-triazole, in some cases however, (e.g. for the synthesis of enzyme inhibitors), the 1,5-disubstituted triazole is preferred.[50] In order to obtain exclusively the 1,5-disubstituted 1,2,3-triazoles from organic azides and alkynes, the groups of Fokin and Jia [51] used a ruthenium(II) catalyst (Scheme 1B). This Ru-catalyzed process, RuAAC, exhibits a good scope with respect to both azides and terminal or internal alkynes,[52] and functional group tolerance. In contrast to CuAAC, RuAAC requires more stringent reaction conditions with respect to temperature and solvent,[53,54] which hampers a broad application as a bioconjugation reaction for biologically relevant molecules.



Scheme 1: Cycloaddition Reactions between Azides and Alkynes: (A) Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction [16-18], (B) ruthenium(II) catalyzed cycloaddition [51], (C) the ring strain promoted cycloaddition [44], (D) the ring strain promoted cycloaddition with 4-dibenzocyclooctynol [45], (E) second generation of ring strain promoted cycloaddition [46,47], (F) the tandem [3+2] cycloaddition-retro-Diels-Alder ligation method [49].

Another reaction that is becoming increasingly popular as an attractive click reaction is the Michael addition between thiols and acrylates,[55] acrylamides,[56] vinyl sulfones[57,58] or maleimides[59] to form thioethers (Scheme 2). This Michael addition reaction, which is also called thiol-ene coupling (TEC)

or "thio-click",[60,61] is highly efficient and orthogonal to a wide variety of functional groups. The thiol-ene coupling makes use of the high nucleophilicity of the sulfhydryl moiety and proceeds under physiological conditions. The formed thioether linkage is very stable under physiological conditions and resists a strong basic or acidic environment and is also stable towards reducing agents, however it is of course susceptible towards oxidizing agents.



Scheme 2: Variations of the thiol-ene reaction. (G) Michael addition between thiols and acrylates,[55] (H) thiols and vinyl sulfones,[57,58] (I) thiols and maleimides,[59] (J) thiols and alkenes (R_2 and R_3 have to be electron withdrawing groups).[62-64]

A drawback of the thiol-ene coupling reaction is the sensitivity of the free thiol functionality toward oxidation into a disulfide. This unwanted side-reaction can be overcome by excluding oxygen from the reaction medium or by adding reducing agents, such as TCEP, or by using protected thiol derivatives. Such protecting groups must meet several requirements; they have to be removed quantitatively under mild conditions and the by-products should be non-toxic. Goessl and coworkers successfully protected the thiol functionality as a thio acetate.[65,66] Thio esters are highly sensitive toward mild base and can be cleaved at pH 8.

A detailed discussion on the reaction rate of the thiol-ene coupling reaction was provided by Hoyle et al.[67]. Based on extensive studies by Morgan et al.[68] the influence of both thiol- and ene structure on the thiol-ene free radical reaction rate is directly related to the electron density on the ene moiety. Electron-rich enes react more rapidly than electron-poor enes, e.g. alkene ≈ vinyl ester > allyl ether > acrylate > N-substituted maleimide > methacrylate.[67]

2.4 Polypeptide-based polymers

There is a great interest in the use of functional peptides as building blocks for the synthesis of peptide-based polymers, since these polymers can be applied for the design of drug delivery systems, scaffolds for tissue-engineering and -repair, and as novel biomaterials.[1,69] However, the synthesis of peptide-based polymers imposes several major synthetic challenges. Current methods to synthesize such polymers require the elaborated use of protection groups and/or unstable pre-activated building blocks[70-72] or the polymers have to be synthesized via protein engineering, which can be very challenging.[73] Recently, new methods have been developed to synthesize peptide-based polymers by chemoselective ligation, where, *unprotected* functional peptides are coupled by an orthogonal conjugation method to form polypeptide products. Especially the CuAAC is very suitable, because the formed 1,2,3-triazole moiety, is a mimic of a native peptide bond [40,41]. In view of the scope of this review, native chemical ligation (NCL) for the synthesis of peptide-based polymers is not discussed. A recent review on protein synthesis by NCL is given by Kent.[74]

In 2005, Fan and coworkers[75] developed a protocol for the solid phase synthesis of peptidotriazoles with alternating triazole and amide linkages in the backbone. The first peptidotriazole they synthesized consisted of four repeating units of 4-pentynoic acid and Fmoc-proline azide **1** (Figure 1). First, 4-pentynoic acid was grafted onto the resin with PyBOP/HOBt as coupling reagents. Next, Fmoc-proline azide was clicked in the presence of CuI/ascorbic acid/DIPEA/30% 2,6-lutidine/DMF, followed by the removal of the Fmoc group with 20% piperidine/DMF and the cycle was repeated. After four cycles, the peptidotriazole was cleaved from the resin to yield the desired product **2** in high purity. To demonstrate the versatility of this approach and its compatibility with the common protecting groups used in peptide chemistry, the synthesis was repeated replacing Fmoc-proline azide by side chain-protected tyrosine-, aspartic acid- and lysine building blocks (Figure 1). Also in this case the desired product **3** was obtained in high yield and purity.

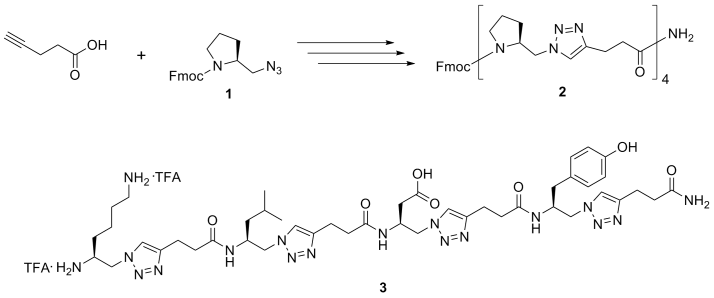
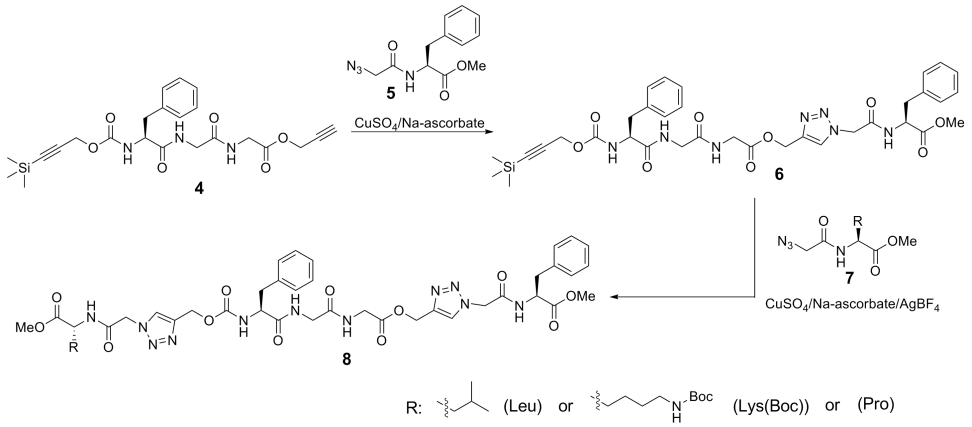


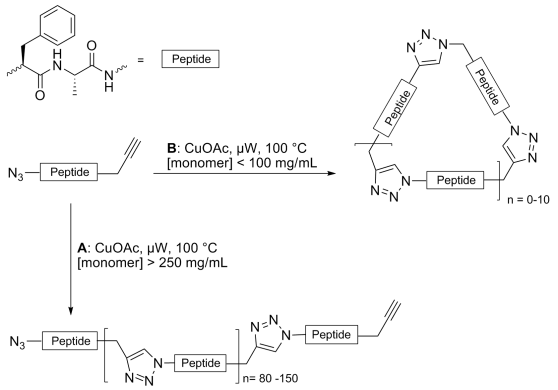
Figure 1: Examples of peptidetriazole sequences synthesized by Fan and coworkers.[75]

Leigh and coworkers[76] developed synthetic methodologies in which the copper(I)-mediated alkyne-azide cycloaddition reaction was combined with a silver(I)-catalyzed TMS-alkyne deprotection[77,78] and this so-called "click-click'' strategy was used to synthesize step-by-step oligomers of peptide triazoles (Scheme 3). The authors started with the tripeptide phenylalanyl-glycyl-glycinate hydrochloride, and functionalized the C-terminus of this peptide with an alkyne and the N-terminus with a TMS-protected alkyne (compound **4**, Scheme 3). In the first reaction they performed a click reaction with azide-containing pseudodipeptides, based on the amino acids phenylalanine **5**, leucine, proline or N^{ε}-Boc-protected lysine. In the presence of $CuSO_4$/Na-ascorbate an almost quantitative yield of mono-clicked product was obtained after 18 h. After the first click reaction the TMS group was removed by treatment with $AgBF_4$. The addition of the second azido pseudodipeptide **7** resulted in the formation of the ditriazole **8** (Scheme 3). Additional $CuSO_4$/Na-ascorbate was needed to drive the reaction to completion. This one-pot method can be a promising tool for peptide ligation reactions and the synthesis of peptide-based polymers.



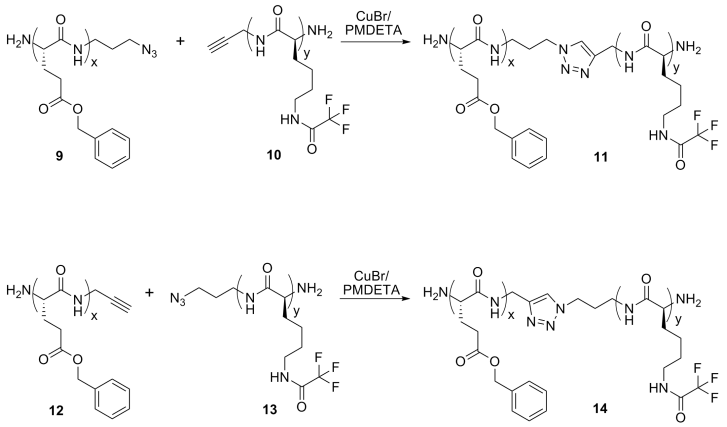
Scheme 3: Synthesis of ditriazole via "click-click'' strategy.[76]

Aiming to develop an efficient method for the synthesis of mimics of natural biopolymers with repetitive peptide sequences, Liskamp et al. explored the polymerization of azido-phenylalanyl-alanyl-propargyl amide as a model peptide under different reaction conditions.[79] As shown in chapter 3 the degree of polymerization was strongly dependent on the polymerization conditions (e.g. type of catalyst, monomer concentration, and reaction temperature) (Scheme 4 approach A and B). In a follow-up study, these optimized polymerization conditions were used to synthesize bio-degradable peptide-based polymers with functional groups, such as the ε-NH_2 group of lysine (chapter 4).[80] In a similar approach Guan and coworkers[81] recently reported on the synthesis of peptide-based polymers that can fold into well-defined β-sheets followed by a self-assembly into hierarchical nanostructures.



Scheme 4: Schematic representation of the synthesis of peptide-based polymers as described in ref. [79]. Approach A: using CuOAc and high concentration of monomer led to predominantly linear polymers. Approach B: using CuOAc and low monomer concentration led to predominantly cyclic oligomers.

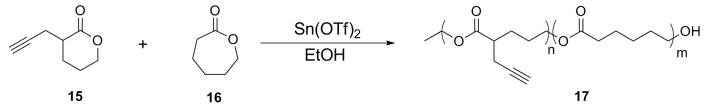
Taton and coworkers[82] developed a methodology for the synthesis of diblock copolypeptides by combining ring-opening polymerization (ROP) of N-carboxyanhydrides (NCA) combined with CuAAC-catalyzed click-chemistry. In their approach, azide- and alkyne-terminated poly(γ-benzyl-L-glutamate) (**9** and **12**, Scheme 5) and poly(N$^{\varepsilon}$-trifluoroacetyl-L-lysine) (**10** and **13**, Scheme 5) were synthesized by ROP of the corresponding NCA with an azide- or alkyne-containing initiator. The azide- and alkyne polymers were subsequently conjugated in DMF at 50 ˚C with CuBr complexed by N,N,N',N'',N''-pentamethyldiethylenetriamine (PMDETA) as catalyst. After 36 h the block copolymers (**11** and **14**) were obtained in almost quantitative yield (Scheme 5).



Scheme 5: Synthesis of diblock copolymers containing poly(γ-benzyl-L-glutamate) and poly(N$^{\varepsilon}$-trifluoroacetyl-L-lysine).[82]

Emrick and coworkers[83] developed a method for the synthesis of biocompatible amphiphilic graft polyesters. In their approach aliphatic polyesters with pendant acetylene groups were synthesized by

ROP of α-propargyl-δ-valerolactone **15** with ε-caprolactone **16** (Scheme 6). These acetylene-functionalized polymers **17** were subsequently grafted with PEG and oligopeptides, using CuAAC and azide-terminated PEG or oligopeptides in the presence of $CuSO_4$/Na-ascorbate for 16 h at 80 °C. The amphiphilic graft polyesters were shown to be biocompatible by *in vitro* cytotoxicity evaluation, suggesting their suitability for a range of biomaterial applications.

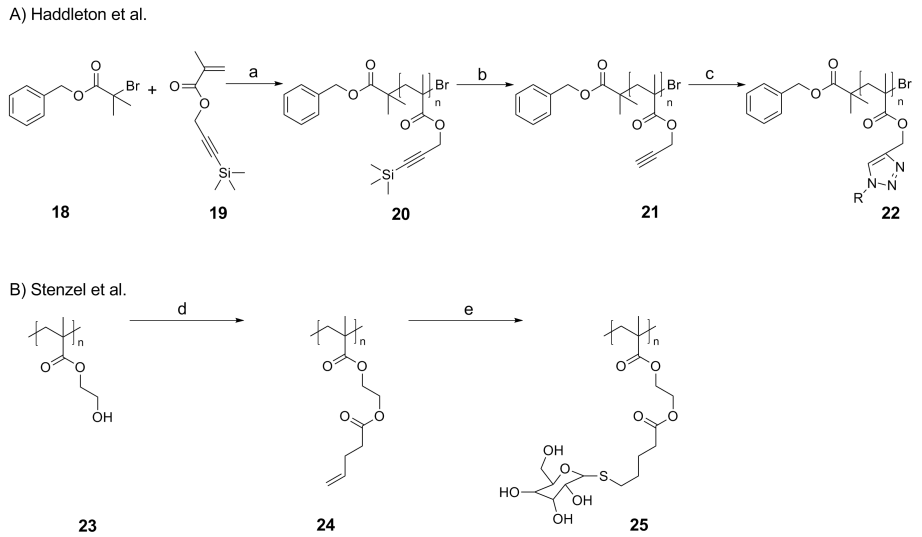


Scheme 6: Synthesis of alkyne functionalized polyesters.[83]

2.5 Glycopolymers

Carbohydrates are involved in a number of important biological processes such as cell-cell recognition, cell-protein interactions and play a role in the targeting of hormones. Moreover antibodies and toxins make use of carbohydrates for selective recognition of their antigens and target receptors.[84-86] However, individual protein-carbohydrate interactions are generally weak, therefore protein-binding carbohydrates exist in higher order oligomeric structures, presenting multiple binding sites, known as the 'cluster glycoside effect'.[87,88] These multiple binding sites help to improve the weak individual interactions to achieve strong and selective interactions. Scientists have attempted to mimic this 'cluster glycoside effect' by synthesizing various well defined synthetic glycopolymers.
Depending on the method applied, such glycopolymers can be divided into two different classes. They can be synthesized either by polymerization of carbohydrate containing monomers (*vide infra*, Reineke et al.[89,90]) or by post-functionalization of polymers with carbohydrates. Especially for the latter approach click chemistry turned out to be a valuable tool.

Several examples to obtain glycopolymers employing both CuAAC and the thiol-ene coupling reaction have been mentioned in the literature. Haddleton and coworkers have synthesized various glycopolymers from alkyne backbone-functionalized polymers and azide functionalized carbohydrates via the CuAAC as shown in Scheme 7A [91,92]. Such as various mannose- and galactose-containing multidentate ligands for studying lectin binding.[91] In this approach, the alkyne-functionalized polymers were synthesized by transition-metal-mediated living radical polymerization (TMM-LRP) of methacrylate **19**. After polymerization, the TMS group was removed with TBAF and acetic acid, followed by the coupling of various protected and unprotected azide-functionalized carbohydrates via CuAAC in the presence of $[CuBr(Ph_3P)_2]$ and DIPEA (Scheme 7A).



Scheme 7: Synthesis of glycopolymers via click reactions. A) Reagents and conditions: (a) N-(ethyl)-2-pyridylmethanimide/CuBr, toluene, 70 °C; (b) TBAF, acetic acid, THF, -20 to +25 °C ;(c) RN_3, $[CuBr(Ph_3P)_2]$, DIPEA; B) (d) 4-pentenoic anhydride, DMP, pyridine, DMF; (e) UV, glucothiose, DMPA, DMF.

Alternatively, Stenzel and coworkers[93] used the thiol-ene coupling reaction for the post-synthesis modification of glycopolymers. First homo- and block copolymer containing di(ethyl glycol) methyl ether methacrylate (DEGMA) and 2-hydroxyethyl methacrylate (HEMA) via RAFT polymerization were synthesized. Then, the homo- and block copolymer were grafted with glucothiose in the presence of the photoinitiator 2,2-dimethoxy-2-phenyl-acetophenone (DMPA) under UV irradiation for 2 h (for homopolymer Scheme 7B). The resulting block copolymer was used to form thermo-responsive micelles that can be used as a potential drug carrier.

In a recent article by Diehl and Schlaad[94], the thiol-ene coupling reaction has been used to functionalize poly[2-(isopropyl/3-butenyl)-2-oxazoline] copolymers with various thiols (e.g. octanethiol, 3-mercaptopropionic acid and 2-mercaptoethanol). Via this approach these authors synthesized a series of glycopolymers with a tuneable cloud point over a wide temperature range.

In a recent article by Haddleton and coworkers[95] the synthesis of alkyne-functionalized polymers via chain transfer polymerization (CCTP) is described. The polymers obtained by this approach can be functionalized via the thiol-ene coupling reaction as well as the CuAAC reaction. In this article, several azide-functionalized carbohydrates were grafted via the CuAAC reaction onto the alkyne side chain of the polymers. The grafted polymers on their turn were end-functionalized using the thiol-ene coupling reaction with different thiols. The synthesized mannose-and galactose- containing glycopolymers were able to function as multivalent ligands for the recognition of lectins. The CCTP

in combination with the thiol-ene coupling reaction and the CuAAC reaction is a powerful tool for the convenient synthesis of many different types of functional polymers and conjugates.

2.6 Polymers for non-viral gene delivery

Gene therapy holds a great promise for the treatment of diseases with a genetic origin that are currently incurable. The success of gene therapy largely depends on the availability of suitable delivery vehicles. Although viral vectors display rather good transfection properties, both *in vitro* and *in vivo*, there are a large number of problems associated with the use of these vectors[96,97]. Therefore, there is an increasing interest in the development of so-called non-viral gene delivery systems [98]. Two major classes of non-viral systems can be distinguished, namely based on cationic lipids or based on cationic polymers. However, generally speaking, the cationic polymers developed to date often display cytotoxicity effects likely due to interactions with proteins and phospholipid membranes. Therefore, new polymers with a significantly reduced cytotoxicity have to be developed. The click reaction can be a valuable tool to synthesize these new polymers for non-viral drug delivery systems.
Reineke and coworkers[89] exploited the CuAAC to synthesize a family of trehalose-based glycopolymers e.g. **26** (Figure 2). They were inspired by the work of Dervan et al., who showed that macromolecules that contain various heterocyclic residues, such as derivatives of pyrrole and imidazole are able to bind nucleic acids.[99] Three polymers, with different amine stoichiometry were synthesized from diazide functionalized trehalose monomers and dialkyne-amide co-monomers in the presence of $CuSO_4$/Na-ascorbate. The polymers contained a trehalose unit for increased biocompatibility, an oligoamine unit for electrostatic interactions with DNA and a triazole functionality for hydrophobic, van der Waals, and hydrogen-bonding interactions with nucleic acids (Figure 2).

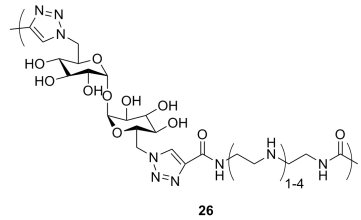


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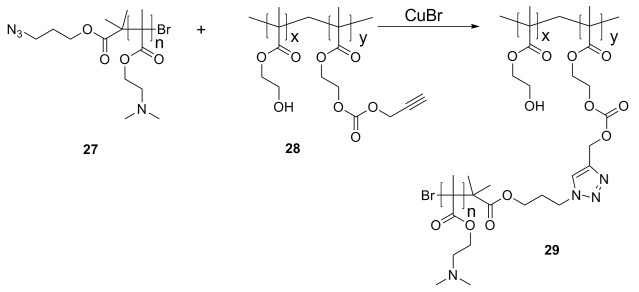
Figure 2: Structure of trehalose polymers synthesized by Reineke and coworkers. [89,90]

All three newly synthesized polymers were effective as nucleic acid carriers in the absence as well as in the presence of serum. The polymer with the highest secondary amine density gave polyplexes with low toxicity and high cellular delivery. The transfection efficiency of the trehalose polymers was an

order of magnitude higher than Jet-PEI, one of the most efficient *in vitro* gene delivery polymers, in serum-free conditions. In a second study [90], the same group investigated the influence of the molecular weight of the trehalose click polymer on polyplex stability and pDNA cellular delivery efficiency. In this study it was shown that a higher degree of polymerization resulted in a higher polyplex stability, although no effect was observed in pDNA binding affinity, cellular uptake, and DNase protection in relation of the M_w.

Ideally, suitable polymeric transfectants should be non-toxic, non-immunogenic, and preferably biodegradable in a controlled manner. Furthermore, biodegradable polymers should yield degradation products with a molecular weight lower than 30 kDa, because these degradation products can be excreted by the kidneys, [100,101] To reduce the cytotoxicity of cationic polymers, Hennink and coworkers[102] designed a high-molecular-weight polymer composed of a low-molecular-weight cationic poly(2-dimetylamino)ethyl methacrylate) (pDMAEMA) that was grafted onto a polymer backbone of an uncharged hydrophilic polymer, poly(hydroxyethyl methacrylate) (pHEMA), via biodegradable linkages. Both pDMAEMA as pHEMA were synthesized by atom transfer radical polymerization (ATRP). For this goal pDMAEMA was end-functionalized with an azide (**27**), while pHEMA was randomly functionalized with acetylene moieties (**28,** Scheme 8). The polymers were "clicked" together via the CuAAC in DMF at 50 °C with CuBr as catalyst (Scheme 8). The molecular weight of the polymer as well as the number of grafts could easily be varied. Upon incubation at physiological conditions (pH 7.4, 37 °C), the carbonate ester bonds were readily hydrolyzed ($t_{1/2}$: 96 h). The molecular weight of the final main degradation product was very close to that of the starting pDMAEMA, indicating that the carbonate esters were quantitatively hydrolyzed. Furthermore, the synthesized polymers were able to condense DNA into small particles, which were able to transfect cells efficiently in the presence of endosome-disruptive INF/7 peptide. Finally, the polymers had a lower toxicity compared to high molecular weight pDMAEMA, making this an effective approach to reduce toxicity of high-molecular-weight cationic polymers.[102]



Scheme 8: Synthesis of degradable-brushed pHEMA-pDMAEMA.[102]

2.7 Dendrimers

Dendrimers are highly branched well-defined polymers, and among others used for the simultaneous presentation of biologically relevant but individually weakly interacting ligands.[103] Multivalent ligands often bind much stronger to the interacting protein than their monovalent counterparts [103-105]. Pieters and coworkers[106] developed a versatile microwave-assisted CuAAC approach that allowed the conjugation of azido carbohydrates with different kinds of alkyne-bearing dendrimers (Figure 3). With this procedure, they were able to synthesize triazole glycodendrimers in high yields, up to the nonavalent level. These glycodendrimers can be used to increase affinities in various applications such as the binding with bacteria, bacterial toxins, and lectins.

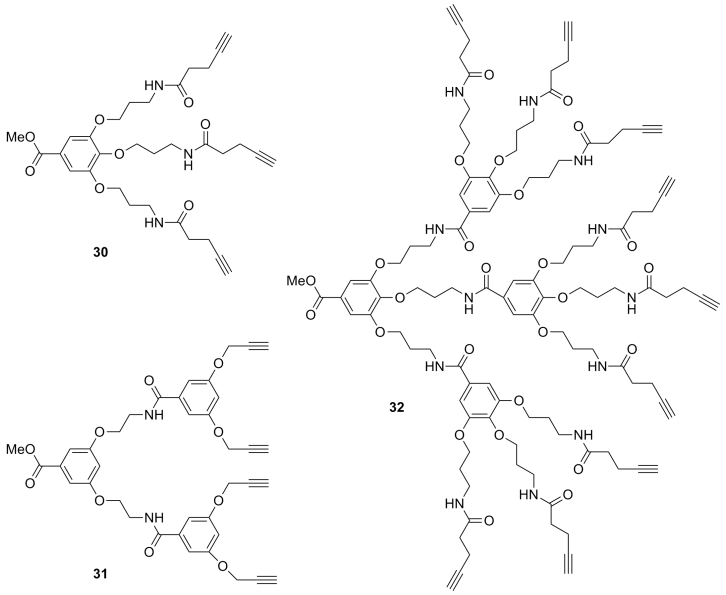
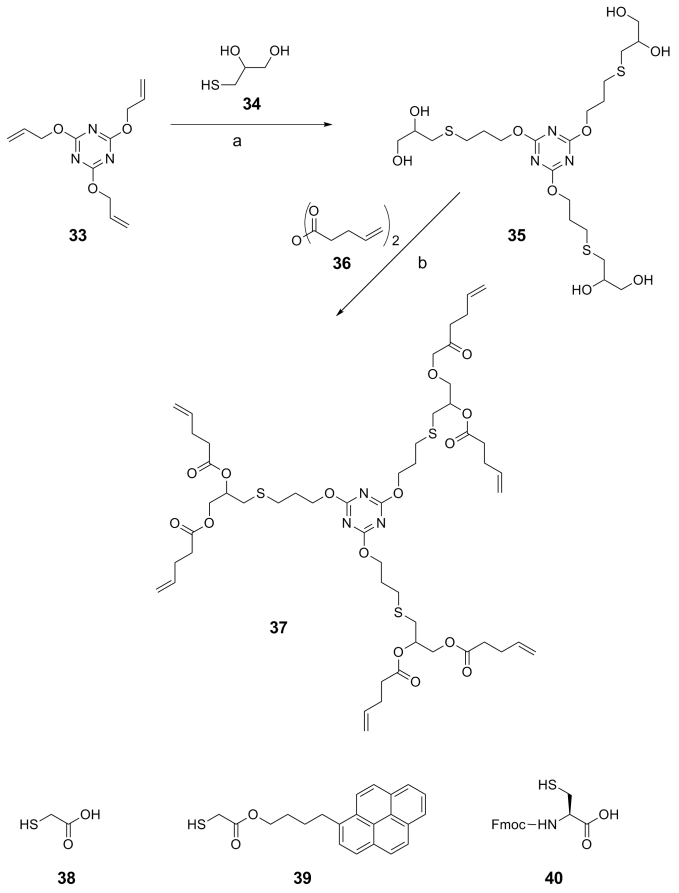


Figure 3: Structures of alkyne-bearing dendrimers used by Pieters, Liskamp and coworkers.[106,107]

In a similar approach, Liskamp and coworkers[107] used the microwave-assisted 1,3-dipolar cycloaddition reaction to synthesize peptide-presenting multivalent dendrimers. The azido-peptides were mixed with the acetylene-functionalized dendrimers (e.g. **31**, Figure 3) in the presence of $CuSO_4$/Na-ascorbate in aqueous DMF and heated at 100 °C in a microwave reactor. The products were obtained in nearly quantitative yield in a relatively short reaction time (5-10 min). With this method, di-, tetra-, octa- and hexadecavalent dendrimeric peptides were successfully synthesized. In a recent study, Liskamp and coworkers[108] used the same strategy as described above to synthesize ^{111}In-labeled DOTA-conjugated dendrimers with multivalent cyclic RGD peptides. The tetravalent ^{111}In-

labeled RGD dendrimers had increased affinity towards the $\alpha_v\beta_3$ integrin receptor and had better tumor targeting properties than their monovalent congeners.

Recently, Hawker and coworkers[109] developed a facile and efficient method for the synthesis and end-functionalization of poly(thioether) dendrimers using the thio-ene coupling reaction. The synthesis of the dendrimers was started from the tris-alkene core 2,4,6-triallyloxy-1,3,5-triazine (**33**, Scheme 9).



Scheme 9: Synthesis of dendrimers via thiol-ene reaction.[109] Reaction conditions: (a) 2,2-dimethoxy-2-phenylacetophenone, *hυ* (30 min), solvent free; (b) DMAP, pyridine.

To this core 1-thioglycerol (**34**) in the presence of catalytic amounts of photoinitiator 2,2-dimethoxy-2-phenylacetophenone was coupled by means of UV-irradiated for 30 min at room temperature to yield the first-generation dendrimer **35**. Subsequent esterification of the hexa-hydroxy dendrimers with 4-pentenoic anhydride **36** yielded the ene-functionalized dendrimers **37**. Repeating the final two reaction steps resulted in the synthesis of dendrimers functionalized with 48 alkene functionalities. End-functionalization was also performed with the thiol-ene coupling reaction with suitable thiol

containing reagents e.g. thioglycolic acid **38**, 4-(pyren-1-yl)butyl 2-mercaptoacetate **39** and Fmoc-Cys-OH (**40)** as shown in Scheme 9.

Waldmann and coworkers[110] used dendrimers and the thiol-ene coupling reaction to immobilize proteins and other biomolecules onto solid surfaces in a patterned way. In detail, they first attached polyamidoamine dendrimers covalently to silicon oxide surfaces (Figure 4 B). The dendrimers were extended with an aminocaproic acid spacer and cystamine was coupled to this spacer and subsequently reduced to obtain a free thiol-functionality (Figure 4 C). Next, the silicon oxide wafers were incubated with terminal-olefin-functionalized proteins dissolved in ethylene glycol and covered with a photomask (Figure 4 D). Subsequent irradiation led to patterning with protein adducts covalently attached via a thioether moiety.

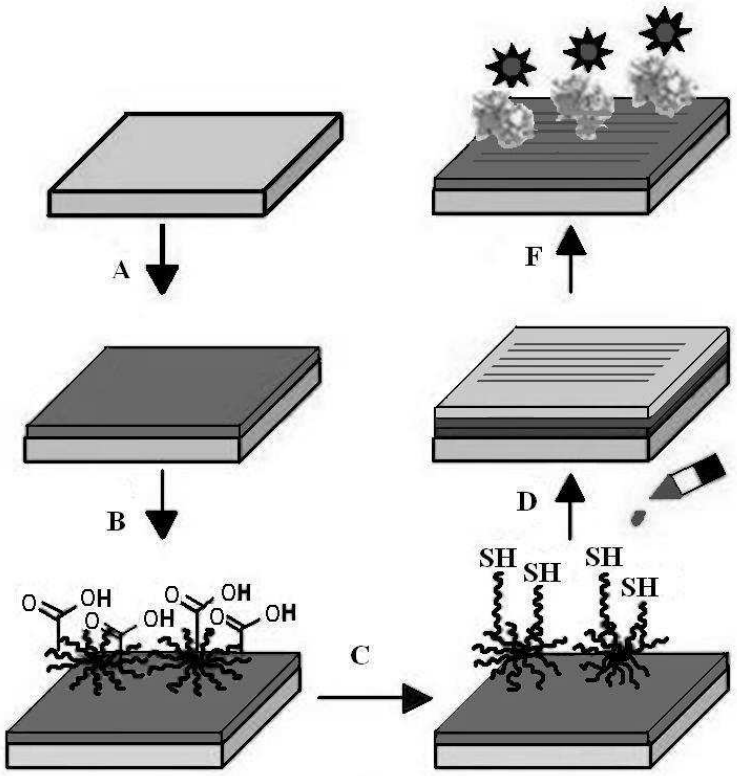


Figure 4: Schematic representation of the approach to immobilize proteins on a solid support.[110] (A) activation of the surface; (B) attachment of dendrimers (aminocaproic acid linker, dendrimers); (C) functionalization with thiols; (D) drop casting of terminal-olefin-functionalized proteins and immediate coverage with a photomask; (E) removal of the mask. (Reproduced with permission from ref. [110])

The amount of protein that was immobilized could be tailored by varying the irradiation time. With this approach, various biomolecules, like biotin, calf-intestine-alkaline phosphatase, the small GTPase Ras, and a phosphopeptide were photochemically attached to the dendrimer-coated wafers. All

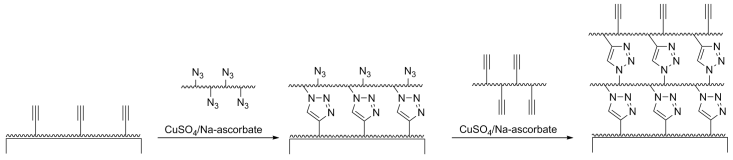
biomolecules were successfully attached and retained their structure and activity. The authors anticipate that this method can be used for the controlled fabrication of structured assemblies of proteins on artificial surfaces.

2.8 Preparation and modification of micro- and nanoparticles

Nanoparticles have great potential in the biomedical and pharmaceutical field[111]. Nanoparticles can for example serve as delivery vehicles for drugs[112,113]. However, current methods to encapsulate drugs in polymeric nanoparticles require the use of organic solvents or harsh conditions, which may result in significant loss of activity, especially if complex biomolecules are used as the drug-like compound. Consequently, novel and mild approaches to obtain nanoparticles are highly desirable.

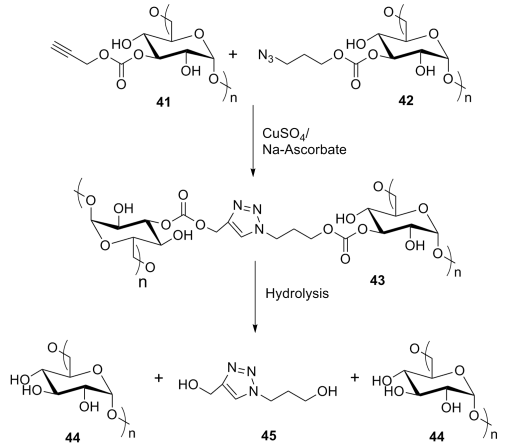
With the aim to prepare functionalized polymersomes from amphiphilic polystyrene-block-poly(acrylic acid), van Hest and coworkers[114] used ATRP to synthesize the block copolymers followed by the substitution of the bromine functional end group of the hydrophilic block by an azide. Upon slow addition of water to a solution of block-co-polymers in dioxane, the amphiphilic block-co-polymers self-assembled into polymersomes with surface-exposed azide functionalities. These azide-functionalized vesicles were used as scaffold for further conjugation with alkyne-functionalized dansyl, biotin and enhanced green fluorescent protein using $CuSO_4$/Na-ascorbate in the presence of the ligand tris-(benzyltriazolylmethyl)amine (TBTA).

Caruso and coworkers developed a general approach for the layer-by-layer assembly of ultra thin, polymer films on planar substrates[115] and particles[116] for the preparation of pH-responsive nanocapsules. In their approach, poly(acrylic acid) with either alkyne or azide functionalities were synthesized by living radical polymerization. Subsequently, the azide and alkyne functionalized poly(acrylic acid)s were alternately assembled on alkyne-functionalized silica particles, with $CuSO_4$/Na-ascorbate as catalyst (Scheme 10). The silica template was removed by treatment with NH_4F-buffered HF at pH 5. The "click capsules" showed pH reversible responsive behavior in a reversible manner, i.e. the capsule diameter varied between about 5 μm in acidic and 8 μm in basic conditions. This pH-responsiveness could be exploited to load and concentrate drugs inside the capsules. Moreover, the presence of azide- or alkyne moieties on the outer shell of the capsules allows the chemoselective post-functionalization of the nanoparticles.



Scheme 10: Schematic representation of the approach described by Caruso and coworkers for the synthesis of ultra thin pH-responsive polymer films. [115,116]

In a similar approach, De Geest and coworkers[117] synthesized dextran-based multilayer films and hollow capsules. By introducing carbonate ester bonds in the azide and alkyne linkages, the multilayers can be degraded by chemical hydrolysis (Scheme 11). In a subsequent article [118], the potential of the biodegradable dextran multilayers for drug delivery was examined. The microcapsules were loaded with fluorescein isothiocyanate- labeled dextran as a model drug compound and incubated at physiological conditions. It was shown that the drug release rate could be tailored by the crosslinking density of the dextran multilayers.



Scheme 11: Synthesis and hydrolysis of dextran-based multilayers.[118]

Schlaad and coworkers[62] used a free radical addition reaction for the synthesis of self assembling peptide hybrid amphiphiles. They grafted cysteine and cysteine-containing dipeptides onto the hydrophobic block of a poly-butadiene-block-poly(ethylene oxide) polymer. The grafting of cysteine had little effect on micelle formation. However, when the dipeptide cysteinyl-phenylalanine was coupled to the amphiphilic polymer, vesicles and worm-like micelles were formed. More recently, Schlaad and coworkers [63,64] used the same method to synthesize polybutadienes modified with hydrophilic functionalities. The free radical addition reaction was used to incorporate thiol-containing molecules, in which the carboxylic acid- or amine functional groups, and amino acid residues were unprotected. With the incorporation of such titratable groups, the polymers were able to self assemble into pH- responsive unilamellar or multilamellar vesicles.

Riguera and coworkers[119] reported an efficient method to synthesize polyion complex (PIC) micelles with the CuAAC catalyzed click reaction that are stable under physiological conditions. In their approach azide-functionalized gallic acid-triethylene glycoside (GATG) dendrimers and PEG-GATG block copolymers were coupled to different alkynes functionalized with either sulfate, sulfonate or carboxylate groups in the presence of $CuSO_4$/Na-ascorbate. When the sulfated PEG-GATG block

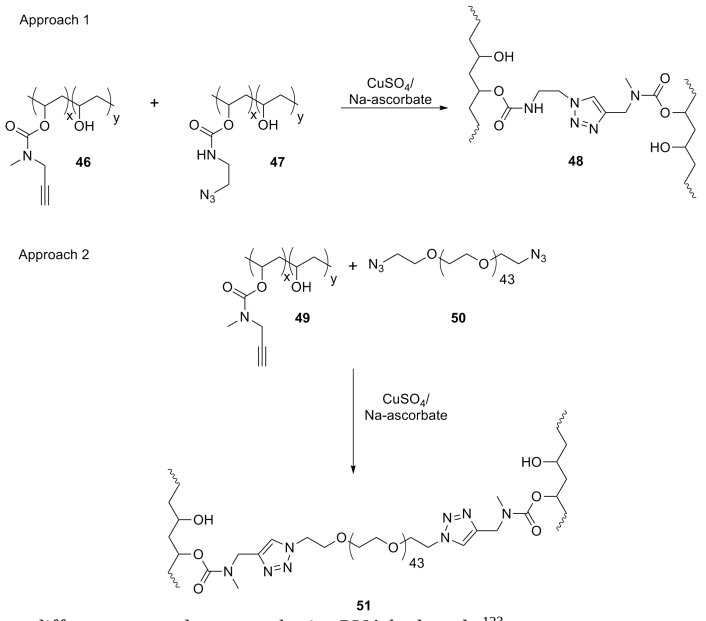
copolymer was mixed in a stoichiometric ratio with poly-L-lysine, spherical micelles with a narrow size distribution were obtained.

In a recent article authored by Pine and coworkers,[120] a new generic method for the covalent linkage of a wide variety of molecules to the surface of colloidal polymer microspheres via the CuAAC reaction has been described. As a typical example, the authors functionalized polystyrene microspheres with two polyethylene oxide-based polymers. It has been stated that this approach is sufficiently general since it can readily be adapted to colloids that consist of other (polymeric) materials. In their approach, polystyrene particles containing 4-vinylbenzyl chloride moieties were converted into the corresponding azides and subsequently reacted with various alkynes to obtain triazole-functionalized microparticles.

In a recent paper by Caruso and coworkers[121], the development of a thiol-ene version of the layer-by-layer assembly of polymer films on silica particles is described. In this approach, poly(methacrylic acid), containing either thiol groups or ene functionalities, were alternatively deposited with poly(vinylpyrrolidone) on silica particles under UV irradiation.

2.9 Hydrogels

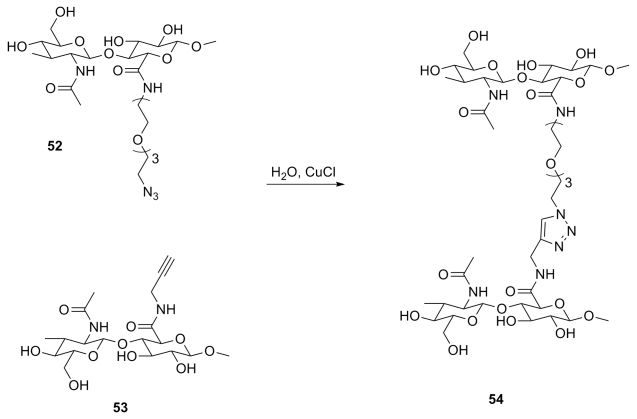
Hydrogels are three-dimensional, hydrophilic polymeric networks capable of absorbing large amounts of water[122]. Hydrogels have received great interest over the past decades, since they can be used for a wide range of applications including drug delivery systems and scaffolds for tissue engineering and repair. In 2006, Hilborn and coworkers were the first to apply click chemistry to synthesize hydrogels.[123] They synthesized poly(vinyl alcohols) (PVA) functionalized with either acetylene or azide groups (**46** and **47**, Scheme 12). Upon addition of $CuSO_4$/Na-ascorbate a hydrogel was formed within a few minutes (**48**, Scheme 12). However, in order to retain the water solubility of the azide- or alkyne-functionalized PVA, only low degrees (1-5%) of modification were possible. In a second approach to synthesize PVA-based hydrogels, acetylene-functionalized polyvinyl alcohol **49** was cross-linked with telechelic bifunctional poly(ethylene glycol) diazide **50** (Scheme 12). This approach was superior in gel formation and gave higher values of gel fraction.



Scheme 12: The two different approaches to synthesize PVA hydrogels.[123]

Hawker and coworkers used PEG as the main structural component for model 'click' hydrogels. In their approach, diacetylene-functionalized telechelic PEG derivatives and tetraazide-functionalized PEG were coupled by 'click chemistry' at room temperature using 2 equiv of PEG diacetylene and 0.4 equiv $CuSO_4$ and 1 equiv of Na-ascorbate. Under these conditions, the hydrogels were formed within 30 min and fluorescence analysis revealed the presence of maximal 0.2% unreacted azide/acetylene moieties. By varying the length of the diacetylene PEG chain they showed that the properties (swelling degree and max true stress) of the hydrogels could be tailored.[124]

Lamanna and coworkers[125] developed a procedure for the formation of hydrogels based on hyaluronan derivatives suitable for tissue engineering applications. They synthesized hyaluronic acid bearing either azide- or alkyne groups (**52** and **53,** Scheme 13). Hydrogels were formed within a few minutes by dissolving both components in H_2O in the presence of 1% w/v of CuCl (Scheme 13). Residual copper entrapped in the hydrogel was removed by dialyzing the hydrogel against an aqueous buffer containing EDTA as metal chelator. The synthesized hydrogels were evaluated for drug-release capabilities with doxorubicin **55** and benzidamine **56** as model drugs (Figure 5). Benzidamine was quantitatively released within hours whereas the release of doxorubicin was prolonged over several weeks. The release rate could be tailored by varying the degree of cross-linking.



Scheme 13: Formation of hyaluronic acid based click-gels. [125]

The slower release of doxorubicin was explained by strong electrostatic interactions between the protonated amino group of doxorubicin and carboxylate groups of the hydrogels. The authors also argued that π-π stacking interactions between the triazole ring and the aromatic moiety likely contributed to the slower release of doxorubicin.

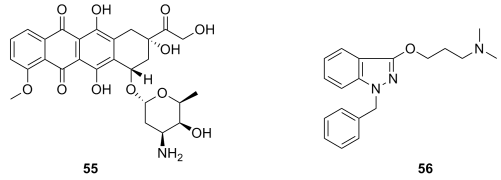


Figure 5: Structures of doxorubicin **55** and benzidamine **56**.

Lamanna and coworkers also examined the suitability of their hyaluronan hydrogels as scaffolds for tissue engineering. Therefore, a hydrogel was formed in a YPD (Yeast Peptone Dextrose) cell suspension and the gels were stored for 2 days at 28 °C. Subsequently, the gels were mechanically broken and the number of cells was counted and 24 h later 80% of the cells exhibited proliferating activity.

Anseth and coworkers[126] developed a procedure to integrate multifunctional photoreactive polypeptides into a hydrogel network. In their approach they combined both the CuAAC and thiol-ene coupling reaction. First, they synthesized hydrogels by clicking tetraazide-functionalized four-arm star-shaped PEG **57** with diacetylene-functionalized peptide **58** (Figure 6). The hydrogels were formed within a few minutes in the presence of 0.25 equiv $CuSO_4$ (based on azide functionality) and Na-ascorbate. Next, a cysteine containing fluorescently labeled peptide was incorporated by thiol-ene photocoupling to the Alloc protecting group present in the hydrogel.

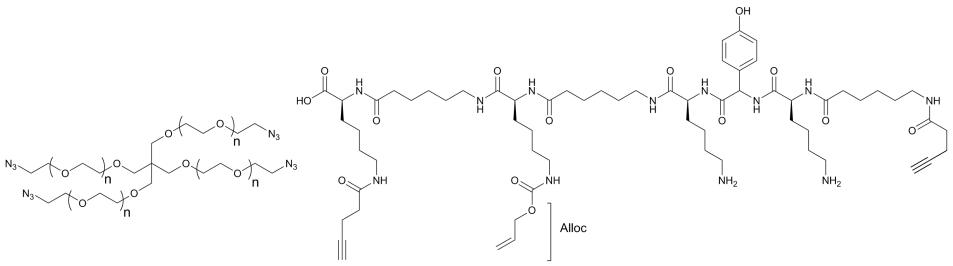


Figure 6: Structure of tetraazide functionalized 4-arm star-shaped PEG **57** and diacetylene functionalized peptide **58.**[126]

Dubois and coworkers[127] synthesized adaptative and amphiphilic polymer co-networks based on hydrophilic poly(N,N-dimethylamino-2-ethyl methacrylate) (pDMAEMA) and hydrophobic poly(ε-caprolactone) (pCL) by a combination of ATRP, ROP and CuAAC. In their approach, the azido-containing 2-(2-azidoethoxy)ethyl methacrylate (AEEMA) was co-polymerized with DMAEMA by ATRP. The p(DMAEMA-co-AEEMA) copolymer was subsequently cross-linked with diacetylene-functionalized pCL, in the presence of CuBr complexed with 2,2'-bipyridine as a ligand at room temperature for 24 h to obtain a highly porous amphiphilic co-network.

Turro and coworkers [128] utilized ATRP and CuAAC for the synthesis of photodegradable polymeric model networks. For the synthesis of the networks, the authors employed two different approaches (Figure 7). In their first approach, linear azido-telechelic macromonomers (MAC) possessing a photocleavable group (nitrobenzyloxycarbonyl) at the center were synthesized by ATRP. Subsequently, both end-groups of the linear polymers were reacted with NaN_3. The obtained azide-functionalized macromonomers were cross-linked with a small tetrafunctional alkyne in the presence of CuBr as catalyst and N,N,N',N'',N''-pentamethyldiethylenetriamine (PMDETA) as ligand. In a second approach, the authors started from a tetrafunctional ATRP initiator containing four photocleavable groups to yield four-armed star-shaped poly(*tert*-butyl acrylate) polymers. Next, the four end-groups were converted into azide functionalities and the star-shaped polymers were cross-linked with a linear bifunctional alkyne. Both model networks degraded upon exposure to UV-light of 350 nm to yield soluble well-defined polymer fragments. In a second paper, Turro and coworkers [129] optimized this procedure by substituting the CuAAC cross-linking with a strain-promoted azide-alkyne cycloaddition, thereby abolishing the need of a (cytotoxic) copper catalyst.

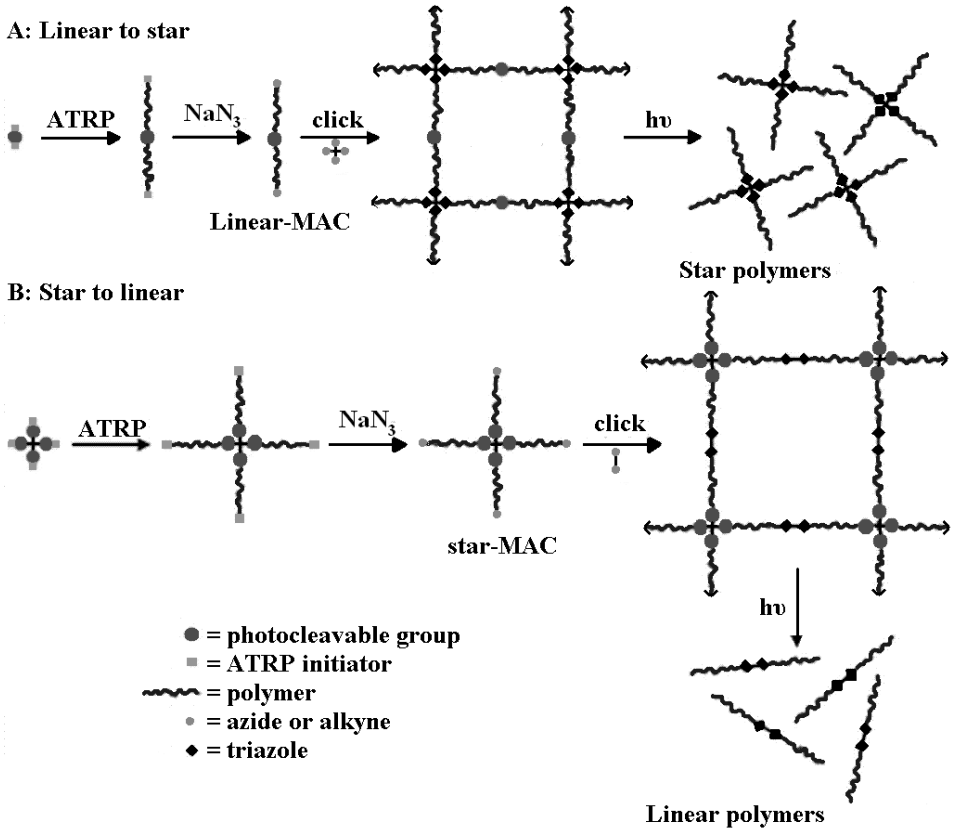


Figure 7: Two different approaches to synthesize photo cleavable hydrogels (Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission [128]).

Zhou and coworkers[130] developed a strategy for the *in situ* gelation of poly(*N*-isopropylacrylamide-co-hydroxyethyl methacrylate) (p(NIPAAm-co-HEMA))-based polymers by the CuAAC. Two (p(NIPAAm-co-HEMA)-based polymers, with either pendant azide or alkyne groups were synthesized. Hydrogels were formed by dissolving the two polymers and incubate them with $CuSO_4$/Na-ascorbate as catalyst and PMDETA as ligand for 24 h at room temperature. The obtained CuAAC hydrogels had faster shrinking/swelling kinetics compared with traditionally synthesized p(NIPAAm) hydrogels.

Yang et al.[131] synthesized PEG-RGD peptide hydrogels for cell entrapment using the CuAAC. PEG is an ideal polymer for hydrogel formation because the formed gels generally have a high water absorbing capacity and are resistant to protein adsorption. However, the viability of encapsulated cells in PEG hydrogels is generally low due to the non-adherent properties for the cells. By virtue of introducing RGD peptides into the PEG hydrogels cell adhesion properties were increased, resulting

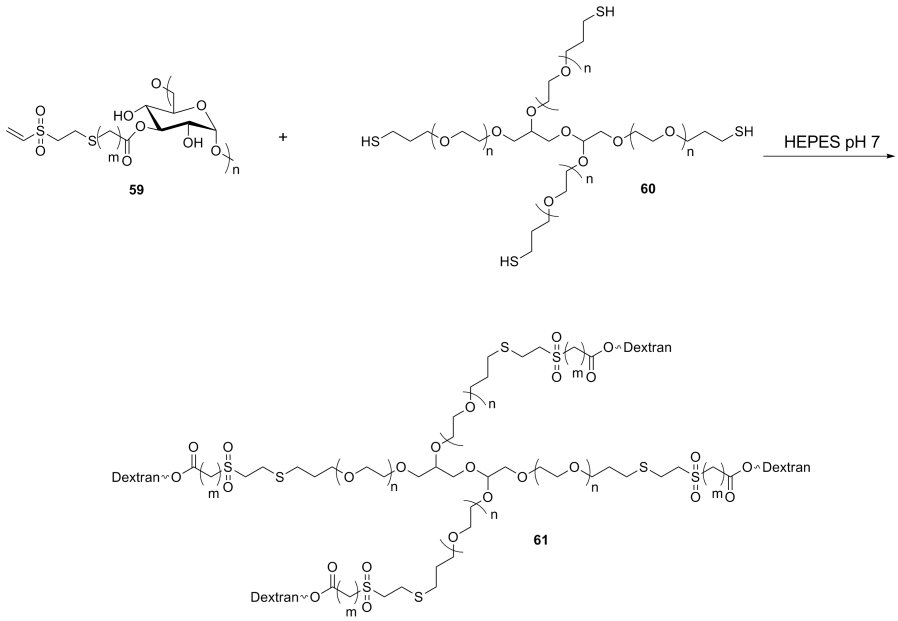
in higher cell viability. For the PEG precursor of the hydrogel, the authors prepared tetra-acetylene PEG by esterification of tetra-hydroxy terminated four-arm PEG with 4-pentynoic acid. As a cross-linking agent they synthesized a series of diazide-functionalized RGD peptides by reacting RGD peptides with 6-azidohexanoic acid. Hydrogels were prepared by combining the acetylene-functionalized PEG and diazide-functionalized RGD peptides in the presence of $CuSO_4$/Na-ascorbate at ambient temperatures. The gelation time could be tailored between 2 to 30 min by variation of the catalyst or precursor concentration and by variation of the temperature. To investigate if the hydrogels could be used as cell delivery vehicle, the hydrogels were seeded with primary human dermal fibroblasts cells. The hydrogels without or at low RGD peptide loading were unable to adhere cells after 18 h. When the RGD peptide loading in the precursor solution was increased, the attachment and proliferation of cells was also enhanced. These data hold promise to use these types of hydrogels as carrier for cell entrapment and tissue engineering.

In 2003, Stein and coworkers[132] reported a method, based on the thiol-ene coupling, to synthesize PEG-based hydrogels *in situ*. They used PEG-based copolymers containing multiple thiol groups synthesized from diamino-PEG and 2-mercaptosuccinic acid. As cross-linking agent, PEG functionalized with vinylsulfone groups was used. Upon combining both PEG polymers, hydrogels with a water content higher than 90% were rapidly formed under mild conditions (pH 6.0 - 8.0 at room temperature). Furthermore, it has been shown that increasing the pH gave rapid gel formation, which is in line with the increasing nucleophilicity of the thiol functionality at higher pH values. The authors have demonstrated that the reaction between thiol and vinylsulfone is compatible with other functional groups present in e.g. proteins by carrying out a release study of fluorescein-labeled BSA, which showed quantitative release after 25 days.

Hubbell and coworkers[133] examined the formation of hydrolytically degradable PEG hydrogels via thiol-ene coupling. They used a four-arm and an eight-arm PEG spacer functionalized with acrylate end-groups and either dithiothreitol or PEG-dithiol as cross-linking agents. The hydrogels were prepared by mixing the acrylate-functionalized star-shaped PEG and dithiol in 1:1 stoichiometric ratio in PBS (pH 7.8) and incubated overnight at 37 °C to ensure complete conversion. The PEG-acrylate contains an ester bond that can be cleaved by chemical hydrolysis. The presence of the thioether group in the proximity of the PEG-acrylate ester greatly increases the sensitivity of the ester toward hydrolysis by several orders of magnitude[134]. By altering the molecular weight of the PEG derivative, or by varying the concentration of either the cross-linker or the PEG-acrylate molecules, hydrogels with different swelling ratios were obtained. The different swelling ratios also resulted in different degradation kinetics.

Feijen and coworkers [135,136] synthesized biodegradable dextran hydrogels by thiol-ene coupling. The dextran hydrogels were formed in situ by mixing solutions of vinylsulfone dextran **59** (Scheme 14)

and tetramercapto four arm star poly(ethylene glycol) **60.** The degradation time of hydrogels **61** could be tailored by varying the length of the spacer of the dextran polymers and the degree of vinylsulfone substitution. To study the versatility of these gels as drug delivery system, four model proteins immunoglobulin, bovine serum albumin, lysozyme and fibroblast growth factor were entrapped inside the gel. The release of the proteins did not show a burst-effect and was depended on the degree of vinylsulfone substitution and dextran molecular weight.



Scheme 14: Dextran hydrogel formation by thiol-ene coupling.[135,136]

Hoffman and coworkers[137] used the thiol-ene coupling method to synthesize heparin-based hydrogels. In their approach, heparin was first reacted with EDC/HOBt and an excess of cystamine to obtain thiol-functionalized heparin. PEG-diacrylate was used as the cross-linking agent. The mechanical properties and gelation kinetics of the hydrogels could be tailored by the degree of thiol group substitution of heparin. The heparin-based hydrogels were degraded at physiological pH by chemical hydrolysis of the ester bond present in the PEG diacrylate cross-linker. To prove that the hydrogels are compatible with cells, the hydrogels were gelated in the presence of fibroblasts. After gelation, most of the cells (95%) retained their viability.

2.10 Conclusions

The CuAAC reaction as well as thiol-ene coupling reaction as discussed in this chapter allow the synthesis of well-defined polymers with tailored properties and provide very attractive possibilities for (bio)conjugation reactions. Several examples of the synthesis and applications of biomedical and pharmaceutical polymers synthesized by the CuAAC-, the thiol-ene reaction or a combination of both reactions were highlighted. In recent years, the CuAAC- and thiol-ene reaction have been used to synthesize polymers with different architectures e.g. block- and graft polymers, dendrimers, and hydrogels, for pharmaceutical and biomedical polymers. Although the CuAAC- and thiol-ene reactions have many advantages they also have some drawbacks. The CuAAC reaction requires the need of a cytotoxic copper catalyst, which is sometimes difficult to remove, while the free thiol needed for the thiol-ene reaction is susceptible to oxidation. The development of new variations of these "click reactions", such as the strain-promoted click reaction, can address the problem of cytotoxic copper catalyst. Nevertheless, both the CuAAC- and thiol-ene reaction have assumed central positions in the synthesis of polymers for biomedical and pharmaceutical applications and it is expected that their importance for the design and synthesis of such polymers will increase rapidly in the coming years.

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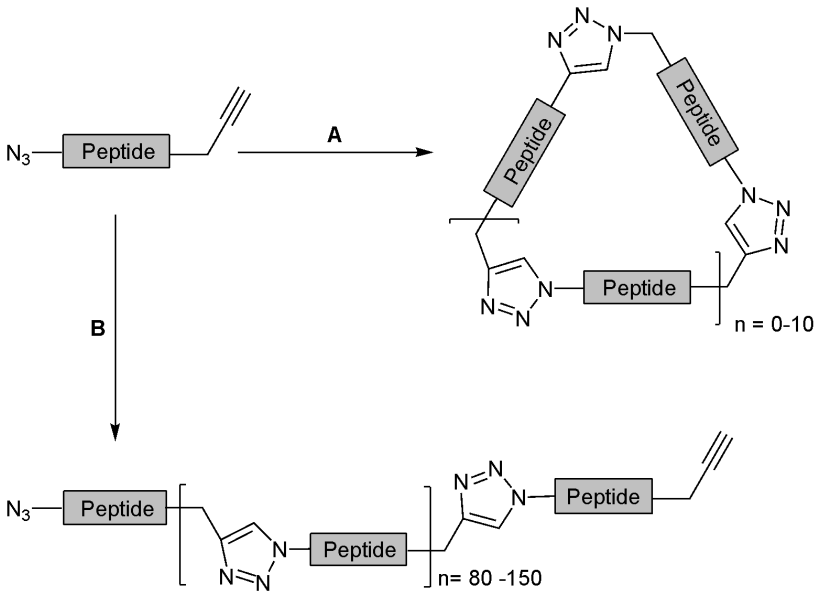
Chapter 3

Synthesis of Peptide-based Polymers by Microwave-assisted Cycloaddition Backbone Polymerization



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3.1 Abstract

This chapter describes a general approach in which model dipeptide azido-phenylalanyl-alanyl-propargyl amide **1** can be efficiently converted into high molecular weight amino acid-based polymers (up to 45,000 Da) by a microwave-assisted 1,3-dipolar cycloaddition reaction. Depending on the reaction conditions, it was found that the outcome of the click reaction could be directed either to large linear polymers (up to 300 amino acid residues) or toward medium-sized peptide macrocycles (4 to 20 amino acid residues). As a proof of principle, a second monomer consisting of N_3-βAla-Pro-Gly-Ser-propargyl amide, representing the repetitive sequence within spider silk was synthesized and polymerized.

3.2 Introduction

There is a great interest in the use of functional peptides as building blocks for the synthesis of peptide-based polymers, since these polymers can be applied for a variety of purposes such as drug delivery systems, scaffolds for tissue-engineering and -repair, and as novel biomaterials.[1,2] Nowadays, the majority of such peptide-based polymers are hybrids of *N*-terminally grafted peptides to a synthetic polymer backbone.[3-5] However, peptide-based biopolymers derive their structural-, mechanical- and biological properties rather from their backbone sequential repetitions than by grafting the peptide sequence to the polymeric backbone. Examples of peptide biopolymers containing repetitive sequences are among others: collagens,[6] elastin and spider silk,[7] antifreeze proteins,[8] mussel glue[9,10] and reflectins.[11]

Current methods for the synthesis of amino acid-based polymers use amino acid-based *N*-carboxy anhydrides,[12-19] for the synthesis of poly-Glu or poly-Lys, activated peptide esters for the synthesis of polymeric elastin models[20] or condensing agents such as diphenylphosphorylazide,[8,21] carbodiimides and acid chlorides.[22]

The reaction between terminal acetylenes and organic azides yielding the corresponding 1,4-disubstituted 1,2,3-triazoles,[23-25] catalyzed by copper (I) seems particularly suitable for chemoselective conjugation reactions. So far, this 1,3-dipolar cycloaddition reaction denoted as 'click reaction' has led to a plethora of applications in the literature.[26-36] Recent studies showed that the 1,2,3-triazole moiety is an effective mimic of a peptide amide bond as present in a β-strand[32] or as dipeptide replacement in α-helical coiled coils.[37] The possibility of chemoselective azide-alkyne coupling in the presence of other (unprotected) functional groups and these topological similarities between peptide amides and 1,2,3-triazoles was the rationale to explore the 1,3-dipolar cycloaddition reaction for the synthesis of peptide-based polymers.[38-44]

As monomer the model dipeptide azido-phenylalanyl-alanyl-propargyl amide **1** (Figure 1) was chosen, since it can be easily synthesized. As a proof of principle, a second monomer azido-βalanyl-prolyl-glycyl-seryl-propargyl amide **2** (Figure 1), representing the repetitive sequence within spider silk was synthesized.

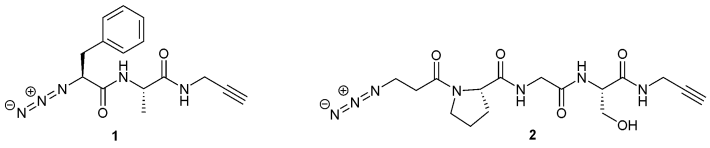
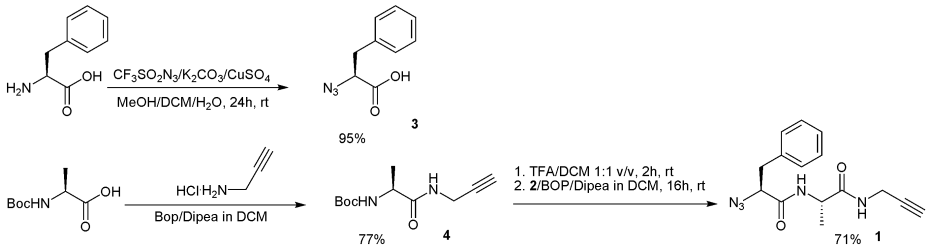


Figure 1: Structure of azido-phenylalanyl-alanyl-propargyl amide (**1**) and azido-βalanyl-prolyl-glycyl-seryl-propargyl amide (**2**).

3.3 Results and Discussion

3.3.1 Monomer synthesis

The monomer azido-phenylalanyl-alanyl-propargyl amide **1**, was synthesized according to Scheme 1. First, azido-phenylalanine **3** was synthesized in 95% yield according to the procedure described by Wong.[45,46] In short a diazotransfer between L-phenylalanine and triflic azide was carried out to yield azido-phenylalanine. Next, *N*-α-(*tert*-butyloxycarbonyl)-alanyl-propargyl amide **4** was synthesized by the coupling of *N*-α-(*tert*-butyloxycarbonyl)-alanine with propargylamine hydrochloride in 77% yield. Subsequently, the *tert*-butyloxycarbonyl protecting group was removed with TFA/DCM and the alkyne was coupled to azido-phenylalanine to yield monomer **1** (71% yield in 2 steps).



Scheme 1. Synthesis of azido-phenylalanyl-alanyl-propargyl amide (**1**).

3.3.2 Polymerization reactions

The initial polymerization reaction was carried out with 200 mg azido-acetylene functionalized dipeptide **1** in 1 mL DMF/H_2O in the presence of $CuSO_4$/Na-ascorbate (entry 1, Table 1). After 3 days of stirring at room temperature, the isolated white precipitate consisted of only small oligomers (up to

tetramers *i.e.* 8 amino acid residues, as was determined by GPC, related to PEG-based molecular weight standards, and LC-MS. Since FTIR still showed a sharp signal at υ 2,100 cm^{-1} of the azide functionality, it was concluded that these were acyclic oligomers. Increasing the dipeptide-monomer concentration to 500 mg in 1 mL DMF/H_2O (entry 2, Table 1) resulted in the formation of a small polymer with a M_n = 6,900 Da (23-mer, 46 amino acid residues). Since formation of copper (I) by the $CuSO_4$/Na-ascorbate redox couple could be the rate-limiting step of the click-polymerization, copper (I) acetate was used instead.

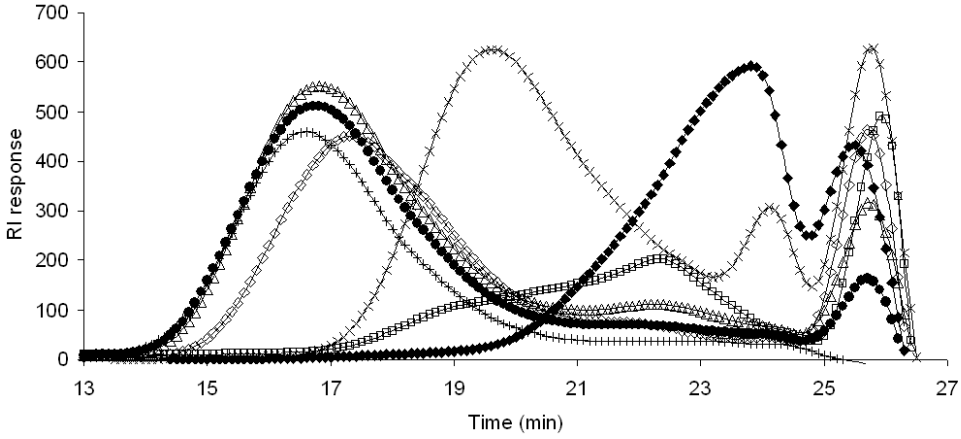


Figure 2: GPC chromatograms from the different polymerization reactions. Entry 1: (♦): up to linear tetramers; Entry 2: (×): small polymers (6,900 Da); Entry 3: (◊): medium-sized polymers (27,600 Da); Entry 4: (Δ): microwave heating: large polymers (38,000 Da); Entry 5: (●): microwave heating and increased concentration: large polymers (44,700 Da); Entry 6: (+): polymerization in the melt (46,700 Da), absence of copper (I); Entry 8: (□): cyclic oligomers.

This led to a fourfold increase of the molecular weight: M_n = 27,600 Da (92-mer, 184 amino acids, entry 3, Table 1). Thus, besides increasing the azido-acetylene peptide monomer concentration, the presence of sufficient Cu(I) in the reaction mixture is a crucial parameter for increasing the molecular weight of the amino acid-based polymer.

Recently,[47,48] the beneficial effect of heating by microwave irradiation on the 1,3-dipolar cycloaddition in order to attach a number of ligands to dendrimeric systems was shown by our group. Therefore, it was expected that further improvement of the polymerization reaction could be achieved in a microwave reactor. Indeed, reaction of **1** at 500 respectively 1,000 mg in 1 mL DMF with CuOAc as a catalyst in a microwave reactor at 100 °C (entries 4 respectively 5, Table 1) gave already

after 30 min a high molecular weight polymer, M_n = 38,000 and 44,700 Da, respectively (149-mer, circa 300 amino acid residues, according to GPC-analysis).

Table 1: Conditions and yields of the different polymerization reactions.

Entry	**1** (mg)	Reaction mixture	T (°C)	Yield (%)	M_n[a] (kDa)	M_w[b] (kDa)	PDI[c]	Tg (°C)
1	200	$CuSO_4$/Na-ascorbate in 1 mL DMF/H_2O (95.5 v/v), 3 days	RT	54	1.1	2.7	1.52	-
2	500	$CuSO_4$/Na-ascorbate in 1 mL DMF/H_2O (95.5 v/v), 3 days	RT	n.d.[d]	6.9	12.7	1.74	-
3	500	CuOAc in 1 mL DMF, 3 days	RT	n.d.[d]	27.6	55.0	1.99	-
4	500	CuOAc in 1 mL DMF, µW (30 min)	100	94	38.0	75.7	1.99	-
5	1000	CuOAc in 1 mL DMF, µW (30 min)	100	92	44.7	77.9	1.96	169
6	Melt	No Cu(I) added	-	100	46.7	86.3	1.84	165
7	Melt	CuOAc	-	100	-	-	-	169
8	50	CuOAc in 1 mL DMF, µW (30 min)	100	77	3.1	8.0	2.61	124
9	500	CuOAc in 1 mL DMF, oil bath (30 min)	100	n.d.[d]	7.0	8.5	1.22	-

[a]M_n (number-average molecular mass) is determined by GPC with 10 mM LiCl in DMF as eluens and PEG standards were used for calibration; [b]M_w (weight-average molecular mass); [c]PDI: polydispersity index; [d]n.d. Yield could not determined due to residual solvent.

Microwave heating not only resulted in an increase of polymer length but also reduced the reaction time considerably. Moreover, the fact that the polydispersity index (M_w/M_n) was near to two was an additional indication for a high conversion rate of the polymerization reaction, according to the Flory equation[49] for typical step polymerizations.

A further, albeit slight, increase in M_n of the polymer (to circa 46,700 Da) was achieved by carrying out the polymerization in the melt in a DSC apparatus (Figure 3), since 1 g in 1 mL DMF (entry 5, Table 1) was close to saturation. Since azido-acetylene peptide **1** was stable up to 250 °C as verified by TGA, polymerization in the melt could be followed by DSC.

First, the polymerization in the melt was carried out in the absence of any copper (I) species (entry 6, Table 1), which clearly showed the melting point of **1** was of 91 °C (solid line, Figure 3). Increasing the temperature (10 °C/min) resulted in a sharp increase of the heat flow due to the exothermic polymerization of **1**. As was indicated above, a high molecular weight polymer, M_n = 46,700 Da (156-mer, > 300 amino acid residues) was formed. However, as polymerization was carried out in absence of copper (I), ^{1}H-NMR analysis clearly showed the presence of both cycloaddition products, for the 1,4-regioisomer: δ_H(triazole): 8.07 ppm, and for the 1,5-regioisomer: δ_H(triazole): 7.41 ppm (both measured in DMSO-d_6).

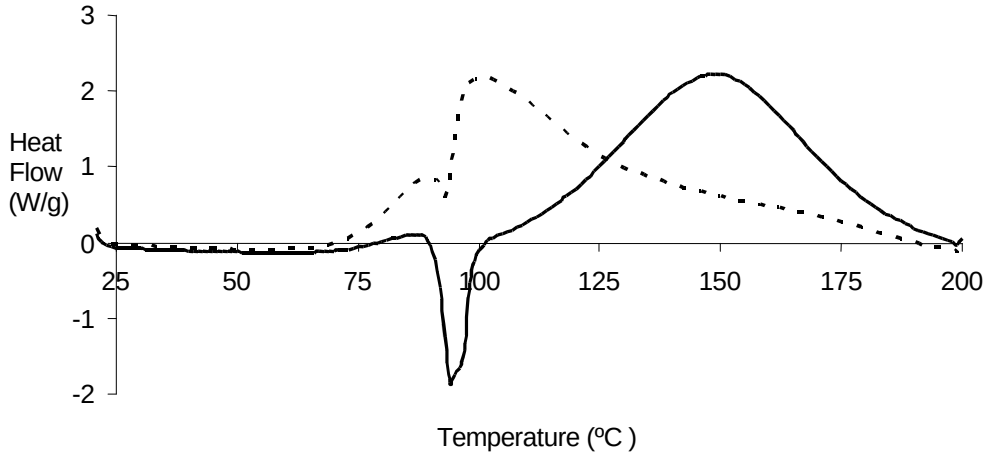


Figure 3. DSC thermograms of the polymerization of **1**. The polymerization was carried out in the melt; without CuOAc (solid line) and with CuOAc (dotted line).

Next, in the presence of CuOAc (entry 7, Table 1) the cycloaddition of **1** took place at a lower temperature and the exothermic reaction was even more pronounced than in the absence of CuOAc (Figure 3). Unfortunately, this polymeric material could not analyzed further due to its insolubility in organic solvents used for GPC analysis (*e.g.* DMF, THF). Thus, the microwave assisted Cu(I)-catalyzed cycloaddition of **1** at a concentration of 1000 mg in 1 mL DMF led to high molecular weight peptide-based polymers containing uniformly a 1,4-substituted triazole linking moiety. Interestingly, when the microwave-assisted Cu(I)-catalyzed click-polymerization was carried out at intermediate concentrations (50–250 mg **1** per mL solvent), GPC-analysis of the isolated polymerization products always showed a bimodal molecular weight distribution (entry 8, Table 1). It was anticipated that the low molecular weight part would contain both cyclic and linear oligomers (Figure 4, reaction conditions **C**). To this end, a sample which was polymerized at 50 mg per mL solvent (entry 8) was fractionated by preparative HPLC and analyzed by FTIR, MALDI-TOF and LC-MS (Figure 5 and 6). Also a second sample (entry 1, 200 mg **1** per mL solvent, Cu(II), RT, reaction conditions **A**) was purified by HPLC and subsequently analyzed. In both samples, the linear and cyclic oligomers could be separated and characterized.

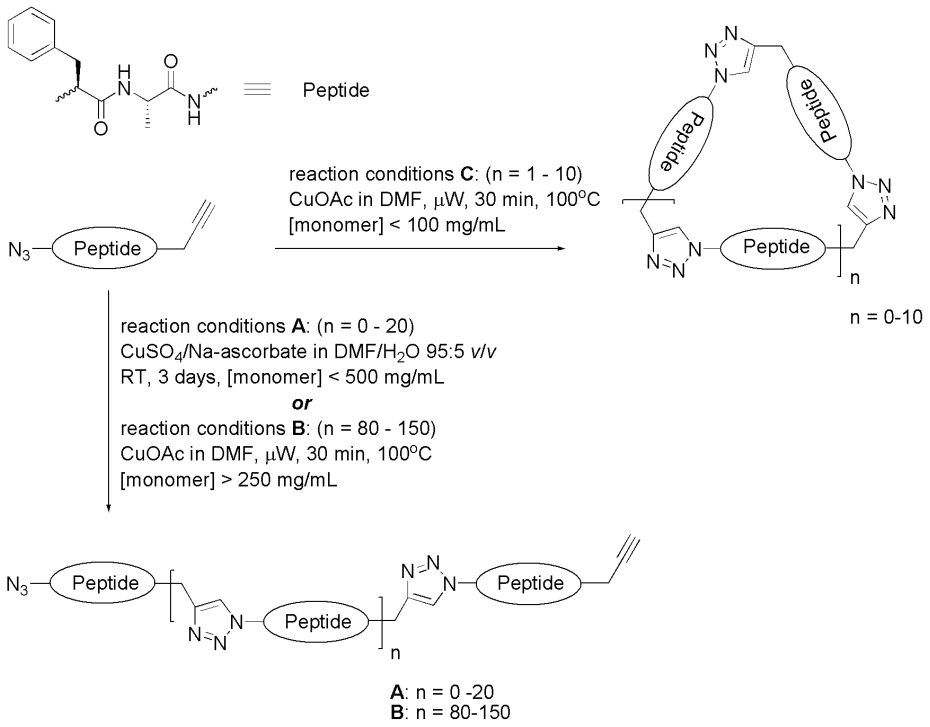


Figure 4. Structures of the synthesized peptide-based polymers. Reaction conditions A and B lead predominantly to linear polymerization products while reaction condition C results in an increased ratio cyclic versus linear oligomer.

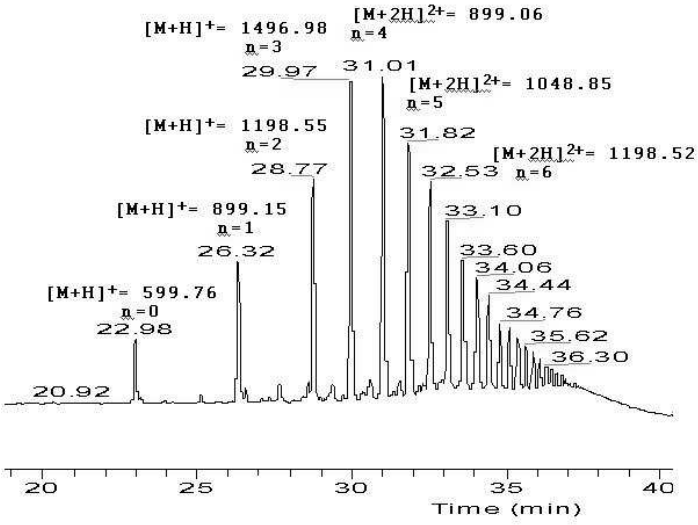


Figure 5. LC-MS pattern of polymerization product from entry 8 (50 mg/mL solvent) showing the cyclic oligomers (n = 0 – 6). Average Δ *m/z* = 299, corresponding to the mass of the dimeric repeating unit.

FTIR in combination with mass spectrometry were used to distinguish between linear and cyclic peptide-based oligomers, since only the linear derivatives showed an intense peak at ν 2100 cm^{-1} corresponding to the azide moiety (Figure 6).

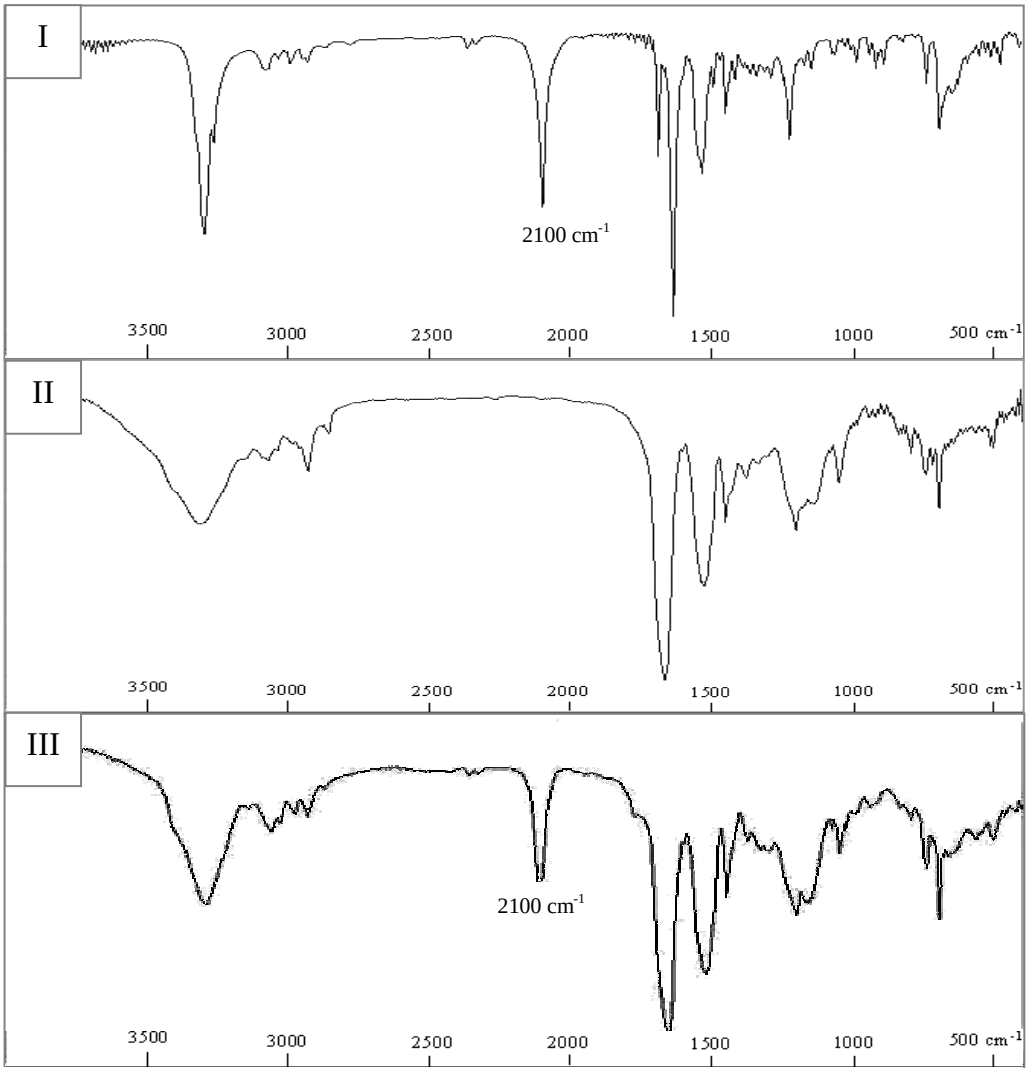


Figure 6. IR spectra of monomer 1 (I), cyclic tetramer as isolated from entry 1 (II) and linear tetramer as isolated from entry 8 (III).

These HPLC analyses made clear that the sample of entry 1 consisted primarily of short linear oligomers while the sample represented by entry 8 consisted mainly of small cyclic oligomers. The smallest cyclic triazole oligomer that was identified contained two dipeptide units (4 amino acid residues, n = 0 in Figure 5).[20] Although the ratio of cyclic versus linear oligomers could be influenced

by choosing the reaction conditions, the exact value of this ratio could not determined accurately. Generally, cyclic oligomers were predominantly formed at low monomer concentrations (50–250 mg **1** per mL solvent, reaction conditions **C**), while at higher concentrations of **1**, preferentially linear oligomers were obtained (reaction conditions **A** and **B**, Figure 4).

In a control experiment, monomer **1** (dissolved in 1 mL DMF, entry 9) was subjected to a Cu(I)-catalyzed click-polymerization which was carried out at 100 °C by using a conventional oil bath. After 30 min a sample was drawn and analyzed by GPC, while the remainder of the reaction mixture was kept at 100 °C. After 2 h respectively 24 h another sample was drawn and analyzed by GPC. It was found that conventional heating did not result in high molecular weight polymers as compared to microwave heating (compare entries 9 with 4). Increasing the reaction time up to 24 h was not effective since a significant increase of the molecular weight of the reaction products was not observed. Apparently, the click-polymerization reaction needs a very efficient heating as is provided by microwave irradiation in the first 30 min to obtain high molecular weight polymers.

3.4 Proof of principle: Spider silk

3.4.1 Introduction

Spider silk is a remarkably strong material. Its tensile strength is comparable to that of high-grade steel, although with a far lower density.[50] These superior mechanical properties of spider silk is derived from their chemical composition. Although there are many different types of spider silk,[51] they share a number of distinguished features. Spider silk is a polypeptide that consists of semicrystalline polymer regions alternated by amorphous domains (Figure 7).

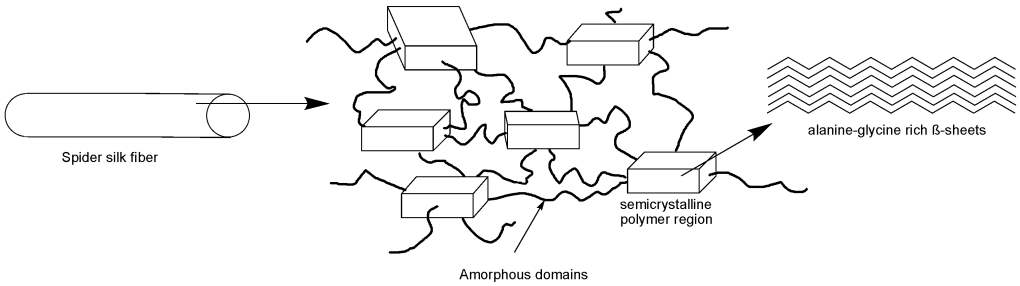


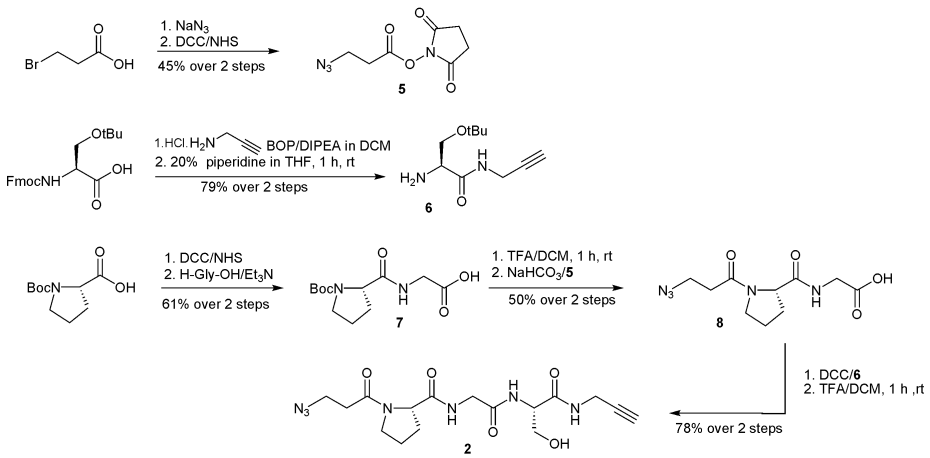
Figure 7. Schematic overview of spider silk fiber.

Four repeating motifs can be distinguished in the majority of sequenced spider silk proteins: 1) polyalanine stretches, 2) glycine-alanine repeats $(GA)_n$, 3) two glycines followed by a variable amino acid $(GGX)_n$, and 4) glycine-proline-glycine followed by a variable amino acid $(GPGX)_n$.[52] The

polyalanine and glycine-alanine repeats $(GPGX)_n$ are known to form crystalline β sheets, while the glycine-proline-glycine stretches form an elastic β-spiral that is similar to the β-turn spiral of elastin.[53] To proof that the optimal polymerization conditions found for the dipeptide monomer **1** can successfully be applied in the polymerization of other peptides, the repeating peptide sequence GPGS was chosen for the next set of polymers. The serine residue was chosen to improve solubility of the polymer. However, the sequence of the peptide was slightly altered for synthetic and stability reasons. First, in order to utilize the click reaction the N-terminus was functionalized with an azide and the C-terminus with an alkyne. Second the first glycine was replaced by β-alanine to reduce the chance of β-elimination. It is known that N_3-Gly-OH might be susceptible for β-elimination during coupling reactions in the presence of a base.

3.4.2 Monomer synthesis and polymerization

The monomer azido-β-alanyl-prolyl-glycyl-seryl-propargyl amide **2**, was synthesized according to Scheme 2. The first building block N_3-β-Ala-ONSu was synthesized starting from bromopropionic acid in 45% yield. The second building block O-*tert*-butyl protected seryl-propargyl amide **6** was synthesized by the coupling of Fmoc-Ser(^{t}Bu) with propargylamine hydrochloride followed by the removal of the Fmoc protecting group with 20% piperidine in THF (79% in 2 steps). The third building block Boc-prolyl-glycine **7** was obtained in 61% yield by the coupling of Boc-proline-OSu and glycine. Subsequently the boc group was removed with TFA/DCM and the dipeptide was coupled to N_3-β-Ala-ONSu to give tripeptide **8** in 50 % yield. Next, azido-βalanyl-prolyl-glycine **8** was coupled to the alkyne **6,** followed by the removal of the *tert*-butyl protecting group with TFA/DCM to yield monomer **2** (78 % yield in 2 steps).



Scheme 2. Synthesis of azido-βalanyl-prolyl-glycyl-seryl-propargyl amide (**2**).

3.4.3 Polymerization reactions

An initial polymerization was carried out with 200 mg of monomer **2** in 1mL degassed DMF/H_2O in the presence of 5 mol-% CuOAc. After 3 days of stirring at room temperature only small oligomers were formed M_n = 6,000 Da (15-mer, 60 amino acid residues according to GPC analysis, related to PEG-based molecular weight standards) (entry 10), which is in line with the previous results found for the dipeptide.

Table 2. Conditions and yields of the different polymerization reactions.

Entry	**2** (mg)	Reaction mixture	T (°C)	Yield (%)	M_n[a] (kDa)	M_w[b] (kDa)	PDI[c]
10	200	CuOAc in 1 mL DMF/H_2O (9:1 v/v), 3 days	RT	87	6.0	7.2	1.20
11	200	$CuSO_4$/Na-ascorbate in 1 mL DMF/H_2O (9:1 v/v), µW (30 min)	100	83	5.9	7.8	1.32
12	50	CuOAc in 1 mL DMF/H_2O (9:1 v/v), µW (30 min)	100	97	5.2	6.2	1.18
13	100	CuOAc in 1 mL DMF/H_2O (9:1 v/v), µW (30 min)	100	92	7.3	8.9	1.22
14	200	CuOAc in 1 mL DMF/H_2O (9:1 v/v), µW (30 min)	100	98	7.5	10.6	1.40
15	500	CuOAc in 1 mL DMF/H_2O (9:1 v/v), µW (30 min)	100	84	8.8	15.7	1.78

[a]M_n (number-average molecular mass) is determined by GPC with 10 mM LiCl in DMF as eluens and PEG standards were used for calibration; [b]M_w (weight-average molecular mass); [c]PDI: polydispersity index.

Therefore the next polymerizations were conducted with the optimal conditions we found for the dipeptide monomer **1**. The reaction mixture was placed in the microwave reactor and irradiated at 100 °C for 30 min. Several concentrations of the monomer (entry 12-15) were used to synthesize polymers with different molecular weights with polymer lengths ranging from M_n = 5,200 Da (13-mer, 52 amino acid residues) till 8,800 Da (22-mer, 88 amino acid residues). These data showed that the polymerization conditions that were found for the dipeptide can be successfully used to synthesize polymers with reasonable M_n. When a high monomer concentration was used (entries 14 and 15), the clear solution was transformed into a turbid gel during irradiation. This gel formation might be the reason why the obtained polymers were significant smaller than the polymers obtained by the polymerization of monomer **1**.

3.4.4 Circular dichroism analysis

Far-UV CD spectroscopy was used for determining the secondary structure of the polymer. Polymer (entry 15) was dissolved in H_2O and a CD spectrum was measured between λ 190 and 240 nm. The CD-spectrum (Figure 8) showed a negative absorption at 205 nm, which indicates that the polymers have a secondary structure. However, from the CD spectrum can be concluded that the synthesized polymers are not able to adopt a β spiral conformation. This might be due to the incorporation of the triazole rings or by the modifications, which were introduced in the monomer.

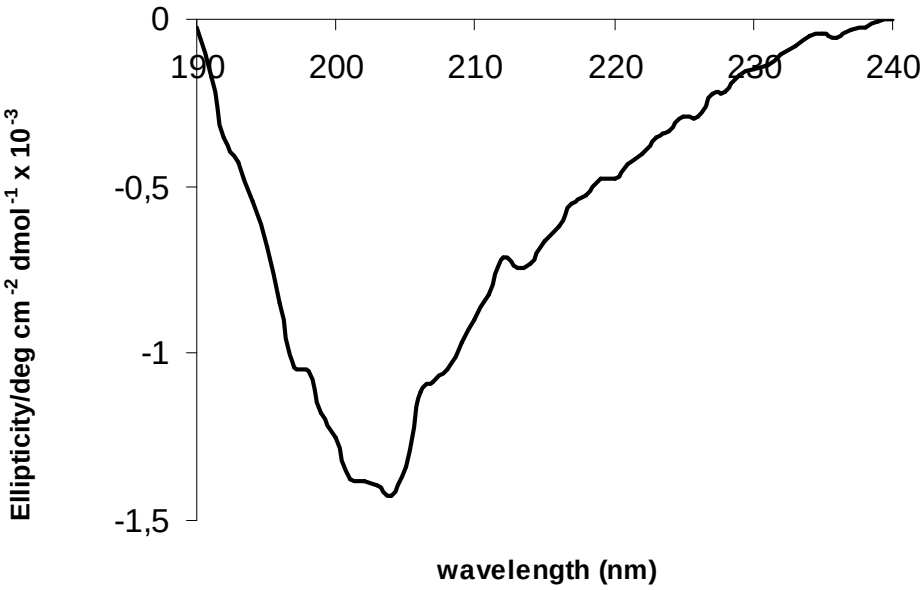


Figure 8. CD spectra of polymer entry 15 in H_2O (1 mg/mL).

3.5 Conclusions

It was shown that high molecular weight amino acid-based polymers could be synthesized via a backbone polymerization connecting their *N*- and *C*- termini employing the Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction. Moreover, the right choice of the reaction conditions was found to be an important factor to obtain either large linear polymers or medium-sized peptide macrocyclic oligomers. These optimized reaction conditions will be applied for the synthesis of biologically relevant peptide-based biopolymers. To obtain high molecular weight polymers, the microwave heating was found to be superior in relation to conventional heating.

3.6 Experimental methods

General procedures: Chemicals were obtained from commercial sources and used without further purification. Reactions were carried out at room temperature unless stated otherwise. Column chromatography was performed with Silica-P Flash silica gel (Silicycle). Thin layer chromatography (TLC) was performed on Merck silica gel 60 F-254 plates. Spots were visualized with UV light, ninhydrin, TDM/Cl_2,[54] $KMnO_4$ or triphenylphosphine/ninhydrin.[55] Microwave reactions were carried out in a Biotage initiator apparatus. ^{1}H-NMR spectra were recorded on Varian Mercury plus 300 MHz or a Varian Unity INOVA 500 MHz spectrometer and chemical shift values (δ) are given in ppm relative to TMS. ^{13}C-NMR spectra (75 MHz) were recorded using attached proton test (APT) pulse sequence and chemical shift values are given in ppm relative to $CDCl_3$ (77.0 ppm). Fourier transform infrared spectra (FTIR) were measured on a Bio-Rad FTS-25 spectrophotometer. Thermogravimetric analysis (TGA) was carried out on a TGA 51 Thermogravimetric analyser (TA Instruments). MALDI-TOF analyses were performed on a Kratos Axima CFR apparatus, with ACTH(18-39) as an external reference (monoisotopic [M + H]+: 2,465.1989 Da) and α-cyano-4-hydroxycinnamic acid as matrix. Elemental analysis was carried out by H. Kolbe Mikroanalytisches Labor (Mülheim/Ruhr, Germany).

Circular dichroism spectroscopy. CD spectroscopy was performed at room temperature in a 0.1-cm quartz cuvette with a dual beam DSM 1000 CD spectrometer (On-Line Instrument Systems, Bogart, GA, USA). The subtractive double-grating monochromator was equipped with a fixed disk, holographic gratings (2400 lines/mm, blaze wavelength 230 nm) and 1.24 mm slits. CD spectra were measured at 1.0 nm intervals in the range of 190-240 nm. Each measurement was the average of ten repeated scans. The spectra were measured in with a final concentration of mg/mL. The measured CD signals were converted to delta molar extinction (Δε).

Gel permeation chromatography. GPC was performed on a Waters 2695 Controller equipped with a refractive index detector (model 2414). The analyses were run on a PLgel MIXED-D column (particle size: 5 μm) (Polymer Laboratories) at 40 °C using 10 mM LiCl in DMF as the mobile phase at a flow rate of 0.7 mL/min. The polymers were dissolved overnight in DMF (containing 10 mM LiCl) at a concentration of 5 mg/mL and filtered through a 0.45 μm filter prior analysis. The samples were analyzed and calibrated using PEG as standards (M: 194-439,600 g/mol; Polymer Laboratories). Peak areas were determined with Empower Software Version 1154 (waters Associates inc.).

Modulated differential scanning calorimetry. (M)DSC was carried out on a Q1000 differential scanning calorimeter (TA Instruments). For the DSC measurement, the samples were heated (10 °C/min) from room temperature to 200 °C. Then, these samples were cooled to 0 °C and subsequently heated (10 °C/min) for a second time to 200 °C. For the MDSC measurement, the sample was heated (2 °C/min) from room temperature to 200 °C. Then, this sample was cooled to 0 °C (2 °C/min) and subsequently heated to 200 °C (rate: 2 °C/min, amplitude: 1.0 °C/min).

HPLC. Preparative HPLC runs were carried out on an Applied Biosystems 400 Semi Automated HPLC System equipped with an Applied Biosystems 757 UV/VIS Absorbance Detector (λ = 214 nm) on an Alltech Alltima C8 column (250 × 22 mm, particle size: 10 μm, pore size: 100 Å) at a flow rate of 10 mL/min using a linear gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95:5 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 100 min. LC/MS(MS) runs were performed on a Finnigan LCQ Deca XP MAX LC/MS equipped with a Shimadzu 10A VP analytical HPLC system. The samples were dissolved in 10% formic acid in CH_3CN/H_2O 1:1 v/v and analyzed using a Phenomenex Gemini C18 column (150 × 4.6 mm, particle size: 3 μm, pore size: 110 Å) at a flow rate of 1.0 mL/min using a linear gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95:5 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 50 min.

Azido-phenylalanine (3): This compound was synthesized according to procedure of Lundquist et al.[45,46] A solution of NaN_3 (18.1 mg 279 mmol) in DCM/H_2O (3:2 v/v) (130 mL) was cooled on ice and triflic anhydride (10.0 g, 35 mmol) was added drop-wise in 2 h. The obtained reaction mixture was stirred for 4 h at room temperature. Subsequently, the organic layer was separated and the water layer was extracted twice with DCM (15 mL). The combined organic layers were washed with 5% $NaHCO_3$ (1 × 25 mL) and triflic azide was immediately used in the next step without further purification.

H-Phe-OH (2.6 g, 15.5 mmol), K_2CO_3 (2.9 g, 21 mmol) and $CuSO_4{\cdot}5H_2O$ (29 mg, 0.11 mmol) were dissolved in $MeOH/H_2O$ (2:1 v/v) (90 mL). Triflic azide in DCM was added and the reaction mixture was stirred for 16 at room temperature. Subsequently, the water layer was separated from the organic layer and acidified with 1N $KHSO_4$ till a pH of 7 was obtained. The water layer was washed with EtOAc (2 × 25 mL) and acidified again with 1N $KHSO_4$ till pH 2. Then the water layer was extracted with EtOAc (3 × 50 mL). The combined EtOAc layers were concentrated in vacuo and the residue was coevaporated with chloroform (3×). Azidophenylalanine was obtained as a yellowish oil in 95% yield. R_f = 0.80 (DCM/MeOH/AcOH 95:5:1 v/v/v). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 2.96/3.16 (dd (J_{ax} 14.1 Hz, J_{bx} 59.8 Hz), 2H, H^{β}), 4.06 (m, 1H, C^{α}), 7.17-7.29 (m, 5H, arom H), 10.72 (broad s, 1H, OH); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: 37.4, 63.0, 127.4, 128.7, 129.2, 135.5, 175.9.

***N*-α-(*tert*-Butyloxycarbonyl)-alanyl-propargyl amide (4):** Boc-Ala-OH (9.1 g, 48 mmol) was dissolved in CH_2Cl_2 (300 mL) and BOP (21.2 g, 48 mmol), DIPEA (25 mL, 144 mmol, 3 equiv) and propargylamine hydrochloride (4.4 g, 48 mmol) were added. After stirring for 16 h, the solvent was removed in vacuo. The residue was redissolved in EtOAc (300 mL) and the solution was washed with 1N $KHSO_4$ (5 × 50 mL), H_2O (1 × 50 mL), 5% $NaHCO_3$ (2 × 50 mL) and brine (1 × 50 mL). The EtOAc layer was dried (Na_2SO_4), filtrated and concentrated in vacuo. The residue was purified by column chromatography with CH_2Cl_2/MeOH (99:1 v/v) as eluent to give **2** as a white solid in 77% yield (8.4 g). R_f = 0.76 ($CHCl_3$/MeOH/AcOH 95:20:3 v/v/v); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 1.37 (d, 3H, H^{β} Ala), 1.46 (s, 9H, $(CH_3)_3$ Boc), 2.22 (s, 1H, C≡CH), 4.05 (d (*J* 6.9 Hz), 2H, CH_2), 4.17 (m, 1H, H^{α} Ala), 4.94 (broad s, 1H, NH urethane), 6.48 (broad s, 1H, NH amide); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: 16.6, 26.7, 27.5, 36.5, 70.0, 78.0, 79.5, 155.4, 171.0.

Azido-phenylalanyl-alanyl-propargyl amide (1): Alkyne **3** (2.37 g, 10 mmol) was dissolved in CH_2Cl_2 (50 mL) and TFA (50 mL) was added. The obtained reaction mixture was stirred for 2 h. Subsequently, the volatiles were removed by evaporation and the residue was coevaporated with toluene (5 ×) and $CHCl_3$ (3 ×) to remove any residual TFA. The residue was dissolved in CH_2Cl_2 (20 mL) and azido-phenylalanine **2** (1.9 g, 10 mmol) followed by BOP (4.4 g, 10 mmol) and DIPEA (5.2 mL, 30 mmol, 3 equiv) were added. The obtained reaction mixture was stirred for 16 h and subsequently concentrated in vacuo. The residue was redissolved in EtOAc (100 mL) and the solution was washed with 1N $KHSO_4$ (5 × 20 mL), H_2O (1 × 20 mL), 5% $NaHCO_3$ (2 × 20 mL) and brine (1 × 20 mL). The EtOAc layer was dried (Na_2SO_4), filtrated and concentrated in vacuo. The residue was purified by column chromatography to give the title compound **1** as a white solid in 71% yield (2.13 g, 7.1 mmol). R_f = 0.61 ($CHCl_3$/MeOH/AcOH 95:20:3 v/v/v); m.p.: 91 °C; FTIR (KBr) υ: 2100 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 1.27 (d (*J* 6.8 Hz), 3H, $H^{ß}$ Ala), 2.23 (s, 1H, C≡CH), 3.06-3.30 (dd (J_{ax} 13.9 Hz, J_{bx} 121 Hz), 2H, $H^{ß}$ Phe), 4.02 (m, 2H, CH_2, CH_2C≡CH), 4.19 (m, 1H, H^{α} Ala), 4.48 (m, 1H, H^{α} Phe), 6.78 (s, 1H, NH Ala), 6.88 (d (*J* 7.3 Hz), 1H, NH Phe), 7.22-7.35 (m, 5H, arom H). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: 11.2, 22.5, 31.6, 41.8, 58.2, 65.0, 72.3, 120.6, 121.9, 122.6, 128.9, 162.0, 164.5; Anal. Calcd for $C_{15}H_{17}N_5O_2$: C 60.12%, H 5.72%, N 23.40%, found: C 60.04%, H 5.65%, N 23.24%.

Azido-βAla-OSu (5). Bromopropionic acid (13.5 g, 82 mmol) and NaN_3 (8.9 g, 137 mmol) were suspended in dry CH_3CN (36 mL). The reaction mixture was refluxed for 4 hours (90 °C). Subsequently, DCM (460 mL) was added and the mixture was washed with 1N $KHSO_4$ (1 × 50 mL). The organic layer was dried (Na_2SO_4), filtered, concentrated in vacuo and coevaporated with $CHCl_3$

(2×). Azido-βAla-OH was obtained as brown oil in 76% yield (7.0 g). R_f = 0.45.(hexane/EtOAc 1:1 v/v). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 2.65 (t (*J* 6.3 Hz), 2H, N_3CH_2), 3.60 (t (*J* 6.3 Hz), 2H, $C\underline{H}_2COOH$), 9.25 (br s, 1H, COOH). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 33.7, 46.4, 177.0.

Azido-βAla-OH (7.0 g, 61 mmol) was dissolved in CH_3CN (400 mL) and N-hydroxysuccinimide (7.1 g, 62 mmol) followed by DCC (12.8 g, 62 mmol) were added. The obtained reaction mixture was stirred for 16 h at room temperature. Subsequently, the reaction mixture was filtered over celite and the solvent was removed in vacuo. The residue was redissolved in DCM (400 mL) and washed with Brine (1 × 100 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated in vacuo. Azido-βAla-OSu was obtained as white solid in 69% yield (8.09 g). R_f = 0.81 ($CHCl_3$/MeOH/AcOH 95:20:3 v/v/v). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 2.86 (s, 4H, CH_2 ONSu), 2.90 (t (*J* 6.6 Hz), 2H, N_3CH_2), 3.69 (t (*J* 6.6 Hz), 2H, $C\underline{H}_2COOH$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 25.4, 30.8, 45.8, 166.3, 168.9.

H-Ser(tBu)-propargyl amide (6). Fmoc-Ser(tBu)-OH (3.83 g, 10 mmol) was dissolved in DCM (150 mL) and BOP (4.6 g, 10.5 mmol), DIPEA (5.2 mL, 30 mmol) and propargylamine hydrochloride (0.92 g, 10 mmol) were added. After stirring for 16 h, the solvent was removed in vacuo. The residue was redissolved in EtOAc (200 mL) and the solution was washed with 1N $KHSO_4$ (3 × 50 mL), H_2O (1 × 50 mL), 5% $NaHCO_3$ (3 × 50 mL) and brine (1 × 50 mL). The EtOAc layer was dried (Na_2SO_4), filtrated and concentrated in vacuo. Fmoc-Ser(tBu)-propargyl amide was obtained as a white solid in 100 % yield (4.2 g). R_f = 0.79 (DCM/MeOH 95:5 v/v). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.21 (s, (H, CH_3), 2.24 (t (*J* 2.6 Hz), 1H, C≡CH), 3.37 (t (*J* 8.5 Hz), 1H, H^{β}), 3.83 (m, 1H, H^{β}), 4.07 (m, 2H, $C\underline{H}_2C{\equiv}CH$), 4.23 (m, 2H, H^{α} (1H), CH Fmoc (1H), 4.41 (m, 2H, CH_2 Fmoc), 5.72 (br s, 1H, NH urethane), 6.98 (br s, 1H, NH amide), 7.26-7.78 (m, 8H, arom H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 27.3, 29.1, 47.1, 54.2, 61.6, 67.0, 71.7, 74.3, 79.1, 119.9, 125.0, 127.0, 127.7, 141.2, 143.7, 156.0, 170.0.

Fmoc-Ser(tBu)-propargyl amide (4.2 g, 10 mmol) was dissolved in 20% piperidine in THF (100 mL). After stirring for 2 h, the solvent was removed in vacuo and the residue was coevaporated with toluene (3×) and chloroform (3×). The residue was purified by column chromatography with (DCM/MeOH 98:2 v/v) as eluent. H-Ser(tBu)-propargyl amide was obtained as 41 % yield (0.80 g). R_f = 0.33 ($CHCl_3$/MeOH/AcOH 95:20:3). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.12 (s, 9H, CH_3), 2.25 (t (*J* 2.5 Hz), 1H, C≡CH), 2.83 (br s, 3H, NH_3), 3.45-3.60 (m, 3H, H^{α} (1H), H^{β} (2H)), 4.05 (m, 2H, $C\underline{H}_2C{\equiv}CH$), 7.62 (br s, 1H, NH amide).

Boc-Pro-Gly-OH (7). L-Proline (11.8 g, 102 mmol) was suspended in H_2O/Dioxane 1:1 v/v (200 mL) and 1 N NaOH (110 mL) was added, followed by the drop-wise addition of Boc-anhydride (21. 7 g, 100 mmol) dissolved in dioxane (100 mL). After stirring for 16 h, the solvent was partially

removed in vacuo and H_2O (200 mL) was added. The solution was washed with Et_2O (3 × 50 mL) and the water layer was acidified with 1N $KHSO_4$ till a pH of 2 was obtained. Subsequently, the product was extracted with EtOAc (3 × 100 mL). The combined EtOAc layers were dried (Na_2SO_4), filtrated and concentrated in vacuo. Boc-Pro-OH was obtained as a white solid in 96 % yield (20.6 g). R_f = 0.77 ($CHCl_3$/MeOH/AcOH 95:20:3 v/v/v); ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.49 (s, 9H, CH_3), 1.91-2.30 (m, 4H, H^β (2H), H^γ (2H), 3.46 (m, 2H, H^δ), 4.35 (m, 1H, H^α), 11.33 (br s, 1H, COOH).

Boc-Pro-OH (20.6 g, 96 mmol) was dissolved in CH_3CN (600 mL) and N-hydroxysuccinimide (12.2 g, 106 mmol) followed by DCC (20.8 g, 101 mmol) was added. The obtained reaction mixture was stirred for 16 h at room temperature. Subsequently, the reaction mixture was filtered over celite and the solvent was removed in vacuo. The residue was dissolved in EtOAc and precipitated in hexanes. Boc-Pro-ONSu was obtained as white solid in 91 % yield (27.5 g). R_f = 0.86 ($CHCl_3$/MeOH/AcOH 95:20:3 v/v/v); ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.48 (s, 9H, CH_3), 2.00 (m, 2H, H^β), 2.39 (m, 2H, H^γ), 2.84 (s, 4H, CH_2 OSu), 3.49 (m, 2H, H^δ), 4.56 (m, 1H, H^α). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 23.4, 24.8, 25.6, 28.0, 28.3, 31.3, 33.6, 46.2, 49.4, 57.1, 59.1, 81.2, 153.5, 168.8, 172.4.

H-Gly-OH (5.9 g, 80 mmol) was dissolved in H_2O/CH_3CN 1:1 v/v (200 mL) and Boc-Pro-ONSu (25.0 g, 80 mmol) dissolved in dioxane (200 mL) or Et_3N (33 mL, 240 mmol) was added so the reaction-mixture had a pH between 7 - 8.5, till all the Boc-Pro-ONSu had reacted. After stirring for 16 h, the solvent was removed in vacuo. The residue was redissolved in EtOAc (400 mL) and the solution was washed with 1N $KHSO_4$ (3 × 50 mL), H_2O (1 × 50 mL), 5% $NaHCO_3$ (3 × 50 mL) and brine (1 × 50 mL). The EtOAc layer was dried (Na_2SO_4), filtrated and concentrated in vacuo. Boc-Pro-Gly-OH was obtained as a white solid in 61 % yield (13.2 g). R_f = 0.62 ($CHCl_3$/MeOH/AcOH 95:20:3 v/v/v). ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 1.45 (s, 9H, CH_3), 1.87-2.09 (m, 4H, H^β (2H), H^γ (2H)), 2.54 (m, 2H, H^δ), 3.95-4.03 (dd (J_{ax} 4.7 Hz, J_{ab} 18.4 Hz, 1H, H^α Gly), 4.11-4.15 (dd (J_{ax} 4.7 Hz, J_{ab} 18.4 Hz, 1H, H^α Gly). 4.37 (m, 1H, H^α Pro), 7.00 (br d, 1H, NH amide), 8.30 (br s, 1H, COOH). 13C NMR ($CDCl_3$, 75 MHz) δ: 23.1, 28.0, 30.0, 31.0, 46.4, 59.7, 78.6, 153.4, 153.7, 171.2, 172.9.

Azido-βAla-Pro-Gly-OH (8). Boc-Pro-Gly-OH (11.4 g, 42 mmol) was dissolved in TFA/DCM 1:1 v/v (80 mL) and after stirring for 1 h at room temperature the solvent was evaporated in vacuo and the crude product was subsequently coevaporated with toluene (3×) and chloroform (3×), The residue was dissolved in H_2O/CH_3CN 1:1 v/v (200 mL). Subsequently, $NaHCO_3$ (10.5 g, 126 mmol) dissolved in H_2O (100 mL) was added drop-wise till a pH of 8.0 – 8.5 was reached. Then Azido-βAla-OSu (8.9 g, 42 mmol) dissolved in CH_3CN (100 mL) was added drop-wise. After stirring for 16 h, the solvent was removed in vacuo. The residue was purified by column chromatography with MeOH/DCM/AcOH (10:89:1 v/v/v) as eluent to give azido-βAla-Pro-Gly-OH as a white solid in 50

% yield (5.6 g). R_f = 0.52 ($CHCl_3$/MeOH/AcOH 95:20:3 v/v/v); ^{1}H-NMR (DMSO-d_6, 300 MHz) δ: 1.91-2.96 (m, 4H, H^β Pro (2H), H^δ Pro (2H)), 2.62 (m, 2H, $N_3CH_2C\underline{H}_2$), 3.38-3.75 (m, 4H, N_3CH_2 (2H), H^γ Pro (2H)), 4.03 (m, 2H, H^α Gly), 4.58 (m, 1H, H^α Pro), 7.47 (br s, 1H, NH amide).

Azido-βAla-Pro-Gly-Ser-propargyl amide (2). Azido-βAla-Pro-Gly-OH (1.24 g, 4.6 mmol) and H-Ser(tBu)-propargyl amide (0.91 g, 4.6 mmol) were dissolved in CH_3CN (150 mL) followed by DCC (1.01 g, 4.9 mmol). After stirring for 16 h, the reaction mixture was filtered over celite and the solvent was removed in vacuo. The residue was purified by column chromatography with MeOH/DCM/AcOH (10:89:1 v/v/v) as eluent to give azido-βAla-Pro-Gly-Ser(tBu)-propargyl amide as a white foam in 78 % yield (1.6 g). R_f = 0.74 ($CHCl_3$/MeOH/AcOH 95:20:3 v/v/v); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 1.21 (s, 9H, CH_3), 1.90-2.45 (M, 4H, H^γ Pro (2H), H^β Pro (2H)), 2.21 (t (*J* 2.6 Hz), 1H, $_2C\equiv CH$), 2.54-2.75 (m, 2H, N_3CH_2), 3.34 (t (J 8.4 Hz, 1H, H^β Ser), 3.44-3.65 (m, 4H, H^δ Pro (2H), $N_3CH_2C\underline{H}_2$ (2H), 3.81 (m, 2H, $C\underline{H}_2C\equiv CH$), 4.04 (m, 3H, H^α Gly (2H), H^β Ser (1H)), 4.40 (m, 1H, H^α Ser), 4.59 (m, 1H, H^α Ser), 4.59 (m, 1H, H^α Pro), 6.97 (br d (*J* 6.9 Hz, 1H, NH amide Ser), 7.11 (br t, 1H, NH amide), 7.61 (br t, 1H, NH amide Gly). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 24.9, 27.3, 28.3, 29.0, 33.8, 43.4, 46.7, 47.5, 53.0, 60.0, 61.2, 71.1, 74.0, 79.5, 169.2, 170.0, 170.1, 172.5. ESI-MS calcd for $C_{20}H_{30}N_7O_5$: 449.24 found m/z $[M+H^+]$ = 450.62.

Azido-βAla-Pro-Gly-Ser(tBu)-propargyl amide (1.6 g, 3.6 mmol) was dissolved in TFA/DCM 1:1 v/v (60 mL) and the solution was stirred for 60 min at room temperature. Subsequently, the solvent was evaporated in vacuo and the crude product was coevaporated with toluene (3×) and chloroform (3×). The crude product was dissolved in water and lyophilized to yield quantitatively monomer **1** as a white solid (1.4 g); ^{1}H-NMR ($CDCl_3$/CD_3OD , 300 MHz) δ: 2.01-2.24 (m, 4H, H^β Pro (2H), H^γ Pro (2H), 2.26 (t (*J* 2.5 Hz), 1H, C≡CH), 2.62 (m, 2H, $N_3CH_2C\underline{H}_2$), 3.54-3.96 (m, 8H, H^β Ser (2H), H^δ Pro (2H), N_3CH_2 (2H), $C\underline{H}_2C\equiv CH$ (2H)), 4.01 (m, 2H, H^α Glyc), 4.36 (m, 1H, H^α Ser), 4.45 (m, 1H, H^α Pro), 7.74 (m, 2H, NH amide (1H), NH amide Ser (1H)), 8.24 (br t, 1H, NH amide gly). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: ESI-MS calcd for $C_{16}H_{23}N_7O_5$: 393.18 found m/z $[M+H^+]$ = 394.42.

Polymerizations in the presence of $CuSO_4$: Dipeptide monomer **1** was dissolved in DMF/H_2O (95:5 v/v) and $CuSO_4$ pentahydrate (0.02 equiv) followed by Na-ascorbate (0.2 equiv) were added. This reaction mixture was stirred for 3 days at room temperature or heated for 30 min at 100 °C in case of the microwave-assisted syntheses. Then, the reaction mixture was diluted with 0.2 N HCl and the precipitate was collected by centrifugation. The pellet was resuspended twice in 0.2 N HCl. Finally, the resulting pellet was dissolved in AcOH and lyophilized.

Polymerizations in the presence of CuOAc: Dipeptide monomer **1** was dissolved in DMF and CuOAc (0.02 equiv) was added. This reaction mixture was stirred for 3 days at room temperature or heated for 30 min at 100 °C in case of the microwave-assisted syntheses. Then, the reaction mixture was diluted with 0.2 N HCl and the precipitate was collected by centrifugation. The pellet was resuspended twice in 0.2 N HCl. Finally, the resulting pellet was dissolved in AcOH and lyophilized.

Polymerizations with conventional heating: Dipeptide monomer **1** was dissolved in DMF and CuOAc (0.02 equiv) was added. This reaction mixture was heated to 100 °C. After 30 min, 2 and 24 h a small amount of the reaction mixture was collected and diluted with 0.2 N HCl. The precipitate was collected by centrifugation. The pellet was resuspended twice in 0.2 N HCl. Finally, the resulting pellet was dissolved in AcOH and lyophilized.

Polymerizations in the melt: Prior to the heat-induced polymerization the stability of the monomer (**1**) was analyzed by thermogravimetric analysis and it was found that **1** was stable up to 250 °C. Based on these results the polymerization reactions were carried out to a maximum of 200 °C. The initial polymerization was carried out in the absence of Cu(I) catalyst. The (M)DSC analysis made clear that an irreversible exothermal reaction took place after the melting point of **1** was reached in the first cycle (rt → 200 °C). In the second and third cycle no melting point or exothermal reaction was observed, however, a glass transition point (T_g) appeared.

Microwave-assisted polymerization reactions in the presence of CuOAc: In a typical procedure (entry 5), monomer **1** (1,000 mg, 3.34 mmol) was dissolved in N_2-purged DMF (1 mL) and CuOAc (9 mg, 73 mmol, 0.02 equiv) was added. The reaction mixture was placed in the microwave reactor (Biotage) and irradiated at 100 °C for 30 min. The clear solution was transformed into a turbid gel. The gel was dissolved in additional DMF and the reaction product was precipitated with 0.2 N HCl (20 mL) The white precipitate was centrifuged and the pellet was washed with 0.2 N HCl. The obtained solid was dissolved in AcOH and lyophilized, the crude polymer was obtained in 92% yield (920 mg). ^{1}H-NMR (DMSO-d_6, 500 MHz) δ: 1.20 (m, 3H, H^β Ala), 3.34/3.39 (double m, 2H, H^β Phe), 4.28 (m, 3H, H^α Ala/CH_2), 5.55/5.70 (m, 1H, H^α Phe), 7.15 (m, 5H, arom H), 8.08 (s, 1H, C=C*H*, triazole), 8.41 (d, 1H, NH), 8.83 (d, 1H, NH), ^{13}C-NMR (DMSO-d_6, 75 MHz) δ: 18.3, 34.1, 37.5, 48.3, 63.4, 122.0, 126.6, 128.1, 128.7, 136.0, 144.2, 167.1, 171.4; DSC T_g: 169 °C.

3.7 References

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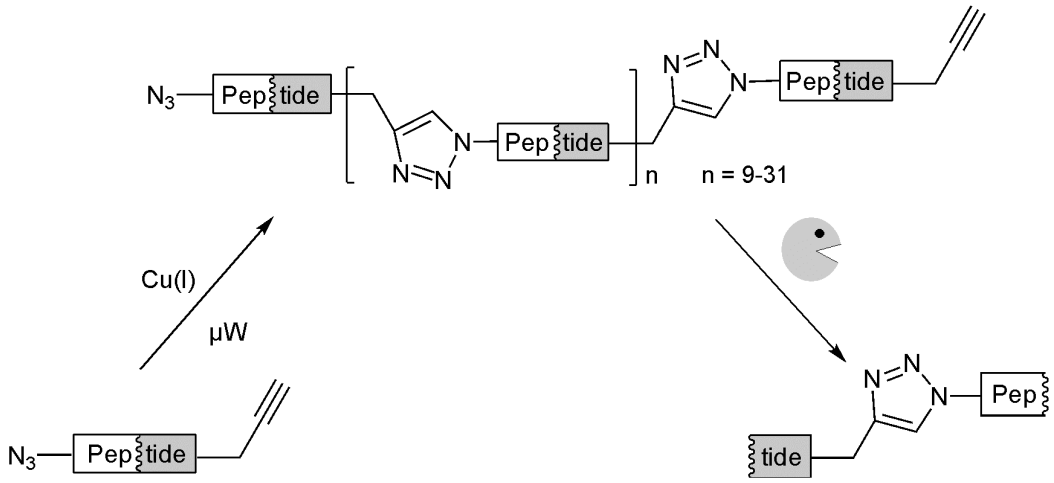
Chapter 4

Synthesis and Characterization of Biodegradable Peptide-based Polymers Prepared by Microwave-assisted Click Chemistry



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4.1 Abstract

In this study, the microwave-assisted copper(I)-catalyzed 1,3-dipolar cycloaddition reaction was used to synthesize peptide triazole-based polymers from two novel peptide-based monomers: azido-phenylalanyl-alanyl-lysyl-propargyl amide (**1**) and azido-phenylalanyl-alanyl-glycoloyl-lysyl-propargyl amide (**2**). The selected monomers have sites for enzymatic degradation as well as for chemical hydrolysis to render the resulting polymer biodegradable. Depending on the monomer concentration in DMF, the molecular weight of the polymers could be tailored between 4.5 – 13.9 kDa (corresponding with 33 to 100 amino acid residues per polymer chain). As anticipated, both polymers can be enzymatically degraded by trypsin and chymotrypsin, whereas the ester bond in the polymer of **2** undergoes chemical hydrolysis under physiological conditions as was shown by a ninhydrin-based colorimetric assay and MALDI-TOF analysis. In conclusion, the microwave-assisted copper(I)-catalyzed 1,3-dipolar cycloaddition reaction is an effective tool to synthesize biodegradable peptide polymers and opens up new approaches towards the synthesis of (novel) designed biomedical materials.

4.2 Introduction

Natural polymers with repeating peptide sequences, such as elastin, spider silk,[1] collagens[2] and mussel glue,[3,4] have received great interest over the past decades, since they can be used to design biomaterials for a wide range of applications including drug delivery systems and scaffolds for tissue engineering and repair. Peptide-based polymers are especially interesting because their primary structure allows the specific cleavage by endogenous proteases,[5] thereby reducing the risk of accumulation and resulting toxic side-effects.[6] However, isolation of these peptide-based polymers from their natural sources is rather difficult and the desire to introduce modifications to modulate their properties necessitates the development of synthetic methods to obtain polymers with repeating peptide sequences in the backbone. The synthesis of peptide-based polymers consisting of amino acid moieties in the backbone imposes several major synthetic challenges. Current methods to synthesize such polymers require the elaborated use of protection groups and unstable pre-activated building blocks such as N-carboxy anhydrides,[7-16] diphenylphosphorylazide,[7,17,18] carbodiimides[5] and acid chlorides.[19]

Recently, new methods have been developed to synthesize peptide-based polymers by chemoselective ligation, where, *unprotected* functional peptides are coupled by an orthogonal conjugation method to form polypeptide products. One of the most extensively used chemoselective ligations is the native chemical ligation (NCL) reaction.[20-22] In NCL an unprotected peptide with a C-terminal thioester

reacts with a peptide containing an N-terminal cysteine to form an amide bond. However, a limitation of NCL is the need for an N-terminal cysteine residue, moreover this cysteine residue is incorporated in the polymer backbone and thereby inducing structural modifications by disulfide formation. To overcome this drawback several other chemoselective amidation reactions[23] like Staudinger ligation[24-26] and azide-thioacid ligation[27-30] have been developed, however, often low yields and moderate reaction rates make these chemoselective reactions unsuitable for polymer synthesis.

Another chemoselective ligation reaction, the copper(I)-catalyzed cycloaddition reaction between terminal alkynes and organic azides yielding the corresponding 1,4-disubstituted 1,2,3-triazoles has been shown to be particularly suitable for polymer synthesis.[31-33] This copper(I)-catalyzed 1,3-dipolar cycloaddition reaction –denoted as the "click reaction"– is compatible with most functional groups present in peptides, abolishing the need for protection groups and is independent of the N- and C-terminal amino acids at the ligation site. In addition, the click reaction can be performed in aqueous solution, has high reaction rates and gives good product yields, making the click reaction very suitable for peptide-based polymer synthesis. Moreover, recent studies showed that the 1,2,3-triazole moiety, which is formed during the click reaction, has clear similarities to the native peptide amide bond in terms of distance and planarity. Therefore it is an effective mimic of a peptide amide bond and has been used as a dipeptide replacement in β-strands and α-helical coiled coils.[34,35] Since its introduction in 2002, the click reaction has found a plethora of applications[36-44] including polymer synthesis. Some examples of applications of click chemistry in polymer synthesis are formation of block copolymers with (ATRP/RAFT and) click chemistry,[45-56] grafting onto a polymer backbone,[57-60] backbone polymerization (polyaddition),[61-67] and polymer networks including hydrogels[68-73] (for review see references [74-78]). However, the number of applications involving the preparation of peptide-based polymers is still very limited. [77,78]

In a previous report[79] it has been shown that the microwave-assisted copper(I)-catalyzed 1,3-dipolar cycloaddition reaction can be used in the backbone polymerization of the model dipeptide azido-phenylalanyl-alanyl-propargyl amide. By varying the reaction conditions it was possible either to obtain small cyclic oligomers (4-20 amino acids) or linear polymers which consisted of up to 300 amino acids residues.[79] To broaden the scope of this polymerization reaction, two novel biodegradable monomers, azido-phenylalanyl-alanyl-lysyl-propargyl amide (N_3-Phe-Ala-Lys-propargyl amide **1**) and azido-phenylalanyl-alanyl-glycoloyl-lysyl-propargyl amide (N_3-Phe-Ala-Glyc-Lys-propargyl-amide **2**) as shown in Figure 1, have been designed and synthesized. The monomers were polymerized with unprotected functional groups (such as the ε-NH_2 group of lysine).

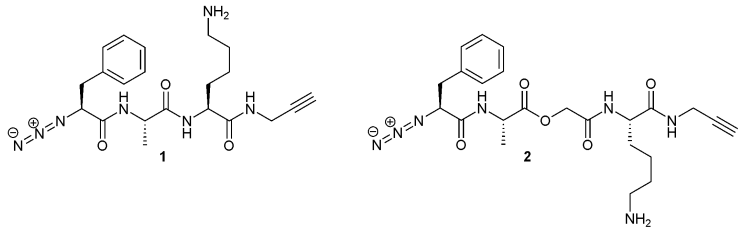


Figure 1: Structures of azido-phenylalanyl-alanyl-lysyl-propargyl amide (N_3-Phe-Ala-Lys-propargyl amide **1**) and azido-phenylalanyl-alanyl-glycoloyl-lysyl-propargyl amide (N_3-Phe-Ala-Glyc-Lys-propargyl amide **2**).

Both monomers have been designed to meet several criteria: the resulting polymers must be water-soluble and contain recognition sites for the model proteases trypsin and chymotrypsin. The lysine residue is a primary recognition element for the protease trypsin, which cleaves the amide bond after the lysine residue, while phenylalanine is recognized by the protease chymotrypsin. Furthermore N_3-Phe-Ala-Glyc-Lys-propargyl amide (**2**) contains an ester bond, which can be cleaved by chemical hydrolysis (Figure 2).

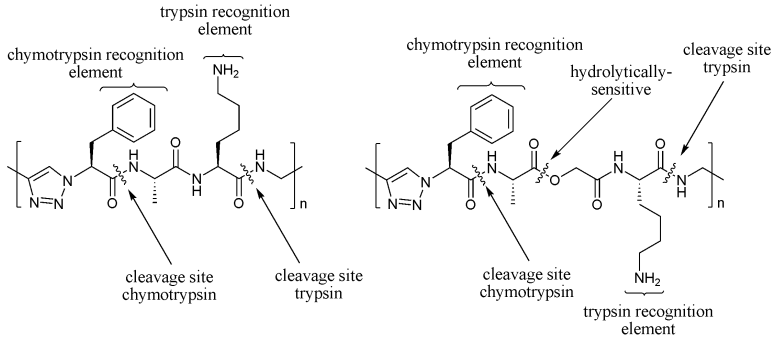


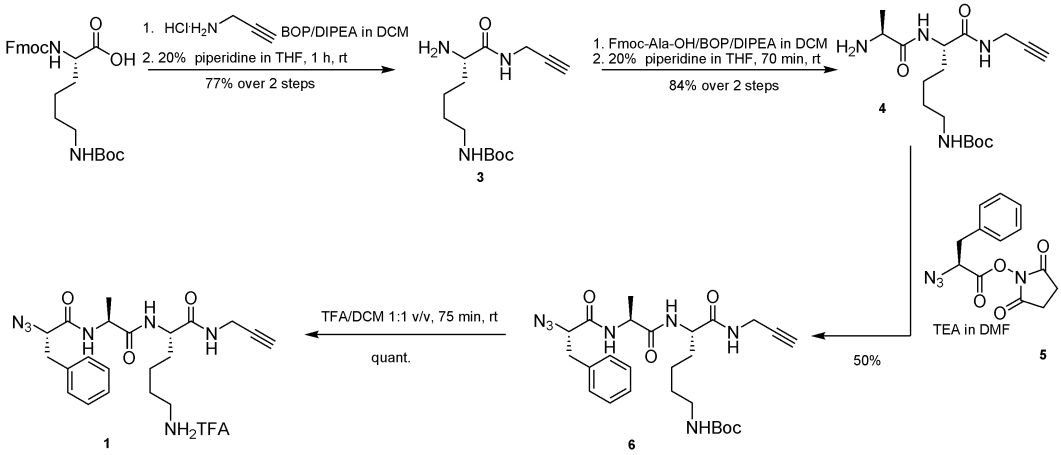
Figure 2: Structures of poly(N_3-Phe-Ala-Lys-propargyl amide) (left) and poly(N_3-Phe-Ala-Glyc-Lys-propargyl amide) (right) with the enzymatic recognition elements for trypsin and chymotrypsin.

4.3 Results and Discussion

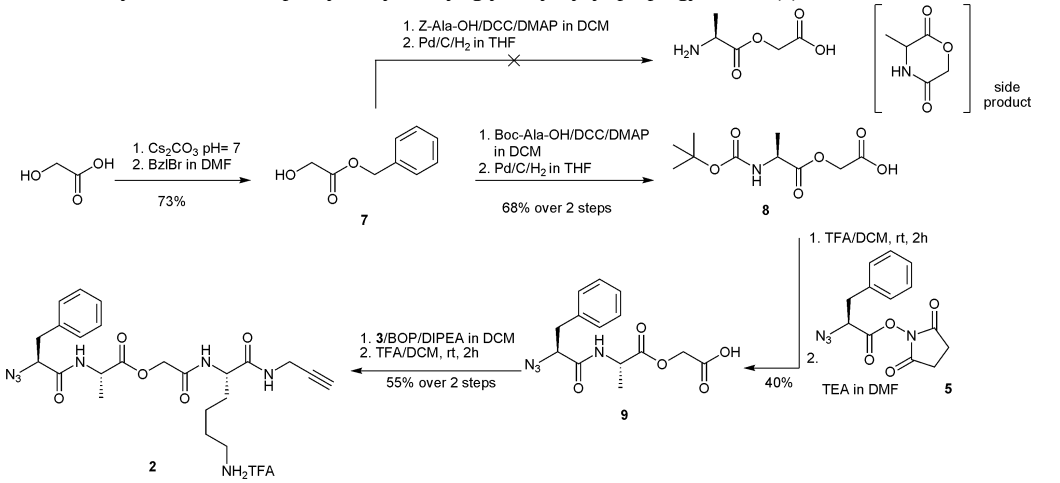
4.3.1 Synthesis and Characterization

The monomers were synthesized according to Scheme 1 and Scheme 2. For monomer **1** all synthesis steps proceeded in reasonable to high yields (Scheme 1). However, synthesis of monomer **2** imposed some challenges. In the first approach, HO-Glyc-OBzl (**7**) was coupled to Z-Ala-OH, since both the Z and the Bzl protection group can be removed by hydrogenation with Pd/C, and the product H-Ala-Glyc-OH was supposed to react with N_3-Phe-OSu (**5**). However, according to NMR, removal of both protecting groups did not result in the formation of H-Ala-Glyc-OH, but the major product was found to be 3-methylmorpholine-2,5-dione (a cyclic depsipeptide) (Scheme 2). To prevent cyclization, Boc-Ala-OH was used; whereafter the Bzl and Boc protection groups were removed separately.

Scheme 1: Synthesis of azido-phenylalanyl-alanyl-lysyl-propargyl amide (**1**).



Scheme 2: Synthesis of azido-phenylalanyl-alanyl-glycoloyl-lysyl-propargyl amide (**2**).



For the polymerization reactions it was decided to use the optimal conditions that were found previously[79]. In short, monomer was dissolved in degassed DMF and 5 mol-% CuOAc was added. The reaction mixture was placed in the microwave reactor and irradiated at 100 °C for 30 min. Several different concentrations of the monomers were used to synthesize polymers with different molecular weights (Table 1). Polymerization reactions using low monomer concentrations resulted in relatively short oligomers, e.g. a concentration of 25 mg monomer **1** in 1 mL DMF (entry 1) gave oligomers of M_n = 4,600 Da, M_w = 5,700 Da (11-mer, ca. 33 amino acid residues according to GPC analysis, related to PEG-based molecular weight standards) and oligomers of M_n = 6,200 Da, M_w = 7,100 Da (13-mer, ca. 50 amino acid residues) for monomer **2** (entry 6). When the monomer concentration was increased, polymers with higher molecular weights were formed, e.g. the reaction of monomer **1** at 400 mg in 1 mL of DMF (entry 5) yielded polymers of M_n = 13,900 Da, M_w =

25,600 Da (33-mer, ca. 100 amino acid residues), while monomer **2** at 500 mg in 1 mL of DMF (entry 10) gave M_n = 9,000 Da, M_w = 14,000 Da (19-mer, ca. 75 amino acid residues) under similar conditions.

Table 1: Polymer Characteristics at Various Monomer Concentrations Used for the Polymerization of Monomer **1** and **2**

Entry	Reaction mixture (in 1 mL DMF)	M_n[a] (Da)	M_w[b] (Da)	PDI[c]	n_n[d]	Yield (%)
1	25 mg **1**	4,600	5,700	1.2	11	93
2	50 mg **1**	4,700	5,900	1.3	11	96
3	100 mg **1**	5,800	8,000	1.4	13	97
4	200 mg **1**	7,900	12,900	1.6	19	98
5[e]	400 mg **1**	13,900	25,600	1.8	33	quant.
6	25 mg **2**	6,200	7,100	1.1	13	89
7	50 mg **2**	6,700	7,900	1.2	14	93
8	100 mg **2**	7,200	8,800	1.2	15	96
9	200 mg **2**	7,700	11,000	1.4	16	92
10[e]	500 mg **2**	9,000	14,000	1.6	19	89

[a]M_n (number-average molecular mass) is determined by GPC with 10 mM LiCl in DMF as an eluent and PEG standards were used for calibration; [b]M_w (weight-average molecular mass); [c]PDI: polydispersity index, [d]n_n: number-average degree of polymerization; [e] these polymers were used in the degradation experiments.

These data showed that the conditions that were found previously[79] can be successfully used to synthesize polymers based on monomers containing unprotected functional groups. FTIR analysis of the polymers showed a significant signal at ν 2100 cm^{-1} of the azide functionality, which opens the possibility to end-functionalize the polymers or to synthesize block co-polymers. Therefore, acetylene-functionalized PEG 2000 monomethylether and an additional amount of CuOAc were added to one of the polymer solutions (entry 5), and the reaction mixture was placed in the microwave reactor and irradiated again at 100 °C for 30 min. After irradiation, according to GPC, the free PEG has reacted and a part of the polymer population showed a clear increase in polymer length due to the incorporation of PEG 2000 (Figure 3). Only a part of the molecular weight distribution increased in molecular weight. It is possible that the remainder of the polymer population mainly consisted of cyclic derivatives, which cannot be extended any further because they no longer present an azide function.

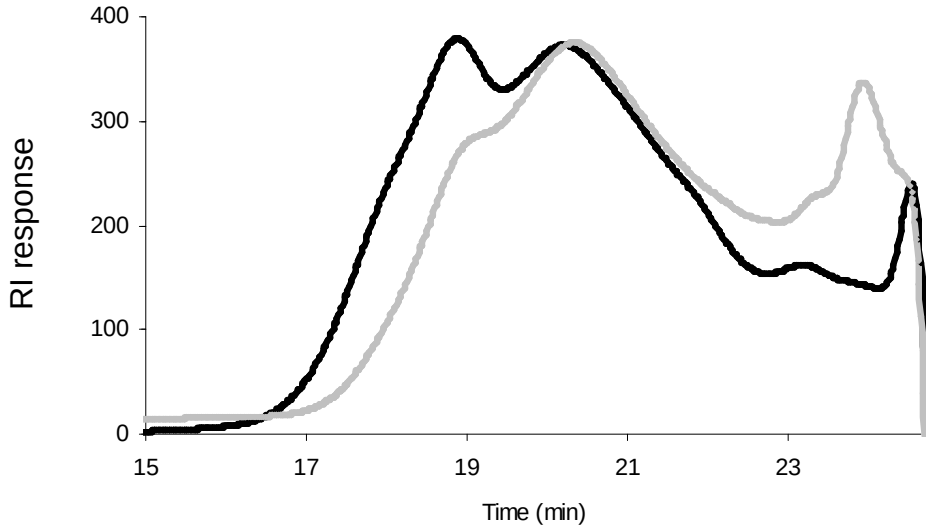


Figure 3: GPC chromatogram of polymer entry 5 (gray) before and after reaction (black) with acetylene-functionalized PEG 2000 monomethylether.

When the polymerization of monomer **1** at 400 mg in 1 mL DMF was performed with an irradiation time of 60 min instead of 30 min, the polymer did not dissolve in common organic solvents (e.g. DMF, DMSO, DMA, chloroform) and could therefore not be analyzed by e.g. NMR and GPC, possibly due to the aggregation phenomena. In further experiments, the soluble polymer of the highest molecular weight was used (Table 1, entry 5 and entry 10). According to thermogravimetric analysis both polymers were stable up to 180 °C.

4.3.2 Monomer Degradation

To get insight into the chemical and enzymatic degradation of the polymers, the degradation of the monomers was studied first. Monomer **1** (N_3-Phe-Ala-Lys propargyl amide) can be likely degraded by trypsin and chymotrypsin because the involved amide linkages are known to be cleaved by these enzymes.[80] In addition, monomer **2** (N_3-Phe-Ala-Glyc-Lys propargyl amide) can be degraded by these enzymes and also by chemical hydrolysis of the ester bond. Since most of the assays with trypsin and chymotrypsin are performed at a pH around 7.4, the hydrolytic stability of monomer **2** was determined at various pH values at 37 °C. The hydrolytic degradation of monomer **2** results in two different fragments, but only the N-terminal fragment (N_3-Phe-Ala-OH) could be detected by LC/MS. The C-terminal fragment (H-Lys-propargyl amide) showed no retention and eluted in the void volume. Figure 4A shows the chromatograms of the degradation products of monomer **2** at different time points at pH 8. In Figure 4B the decrease in monomer **2** is plotted against time for pH 8, and the

monomer concentration decreased according to pseudo first order kinetics (a plot of ln k versus t was linear).

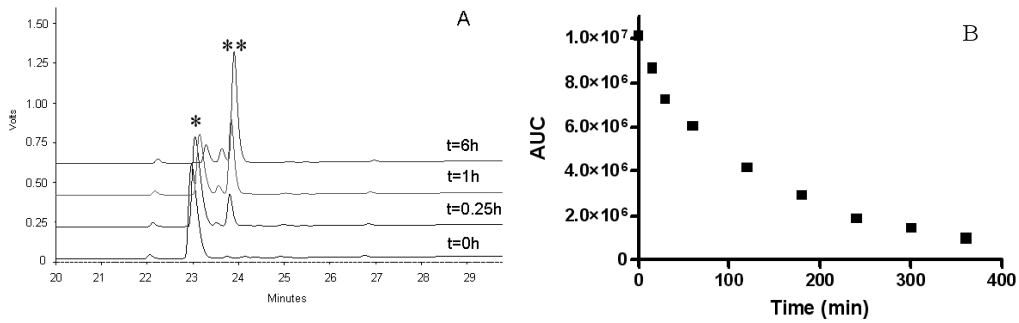


Figure 4: (A) RP-HPLC chromatograms of monomer 2 incubated at pH 8.0 at 37 °C, * = N_3-Phe-Ala-Glyc-Lys-propargyl amide, ** = N_3-Phe-Ala-OH, (t = 0 h), (t = 0.25 h), (t = 1 h) and (t = 6 h); (B). Degradation profile of monomer 2 at pH 8 at 37 °C.

The log k –pH profile in Figure 5 shows that above pH 6, the chemical hydrolysis of the ester bond is specifically hydroxyl-catalyzed, since the slope of the curve approaches 1. The half-life times ($t_{1/2}$) of monomer **2** at pH 7.4 and 8.5 at 37 °C, were 5 and 0.5 h, respectively, which is too fast to perform enzymatic degradation experiments. Since the monomer is relatively stable at pH 5.0 ($t_{1/2}$ ~ 140 h), the enzymatic degradation of monomer **2** was studied at pH 5.0 instead of pH 7.5 to minimize chemical hydrolysis during the enzymatic degradation assays.

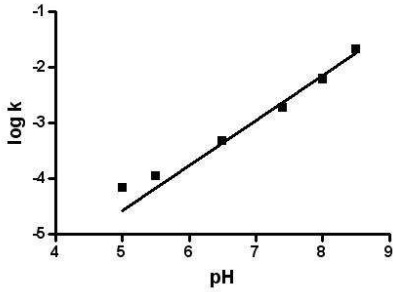


Figure 5: Log k-pH profile of monomer **2.**

It was expected that when monomers **1** and **2** (Figure 1) were incubated with chymotrypsin, the amide bond between the residues phenylalanine and alanine would be cleaved resulting in two fragments, an N-terminal part, N_3-Phe-OH (both peptides) and the C-terminal fragments, H-Ala-Lys-propargyl amide (monomer **1**) and H-Ala-Glyc-Lys-propargyl amide (monomer 2). In case of trypsin, it was expected that the amide bond after the lysine residue would be cleaved resulting in two fragments, an

N-terminal part, N_3-Phe-Ala-Lys-OH (monomer **1**) and N_3-Phe-Ala-Glyc-Lys-OH (monomer **2**) and a C-terminal part, propargyl amine (both peptides). HPLC analysis indeed showed, as was anticipated, that both monomers were cleaved by chymotrypsin and trypsin (Figure 6). The half-life times ($t_{1/2}$) of the monomers are reported in Table 2.

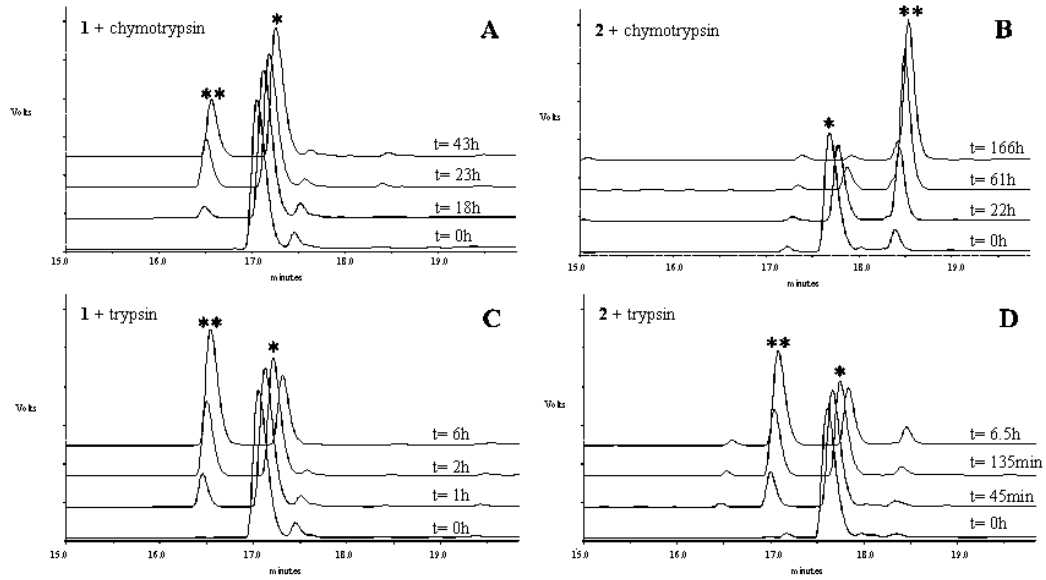


Figure 6: RP-HPLC chromatograms of monomer **1** (N_3-Phe-Ala-Lys-propargyl amide) incubated with chymotrypsin (A) * = N_3-Phe-Ala-Lys-propargyl amide, ** = N_3-Phe-OH, and trypsin (C) * = N_3-Phe-Ala-Lys-propargyl amide, ** = N_3-Phe-Ala-Lys-OH; and monomer **2** (N_3-Phe-Ala-Glyc-Lys-propargyl amide) incubated with chymotrypsin (B) * = N_3-Phe-Ala-Glyc-Lys-propargyl amide, ** = N_3-Phe-OH and trypsin (D) * = N_3-Phe-Ala-Glyc-Lys-propargyl amide, ** = N_3-Phe-Ala-Glyc-Lys-OH.

Table 2: Half-life times ($t_{1/2}$) of monomers 1 and 2 degraded with chymotrypsin and trypsin.

Monomer at 3 mg/mL per experiment	Enzyme	Enzyme concentration	pH	$t_{1/2}$ (h)
N_3-Phe-Ala-Lys-propargyl amide (**1**)	chymotrypsin	0.8 µM	7.5	91
N_3-Phe-Ala-Lys-propargyl amide (**1**)	trypsin	0.016 µM	7.5	1.5
N_3-Phe-Ala-Glyc-Lys-propargyl amide (**2**)	chymotrypsin	0.8 µM	5.0	21
N_3-Phe-Ala-Glyc-Lys-propargyl amide (**2**)	trypsin	0.8 µM	5.0	4.0

4.3.3 Polymer Degradation

The two synthesized polymers, poly(N_3-Phe-Ala-Lys-propargyl amide) and poly(N_3-Phe-Ala-Glyc-Lys-propargyl amide), were designed to contain recognition sites for the proteases trypsin and chymotrypsin. Moreover, the latter polymer also contains ester bonds that can be cleaved by chemical hydrolysis. Therefore, chemical degradation studies of poly(N_3-Phe-Ala-Glyc-Lys-propargyl amide) were carried out in buffer pH 7.0 at 37˚C. MALDI-TOF analysis of the polymer sample shows a clear

shift of the median toward a lower molecular weight distribution after 7 h of incubation (Figure 7), demonstrating that this polymer was indeed degraded by chemical hydrolysis.

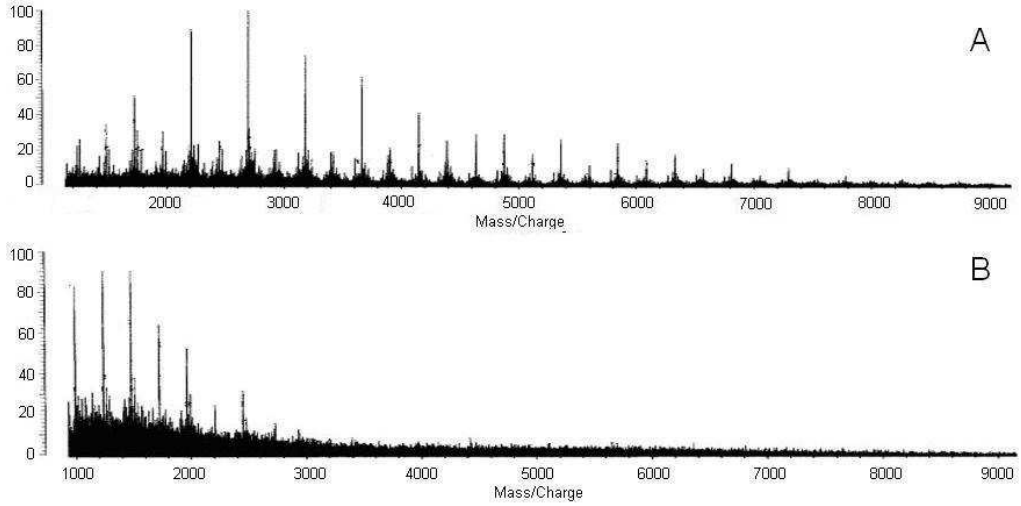


Figure 7: Mass spectra of poly(N_3-Phe-Ala-Glyc-Lys-propargyl amide) (entry 10) incubated in 100 mM phosphate buffer pH 7.0 at 37 °C (A) t= 0 h and (B) t= 7 h.

Chymotrypsin induced degradation of poly(N_3-Phe-Ala-Lys-propargyl amide) and poly(N_3-Phe-Ala-Glyc-Lys-propargyl amide). When amide bonds in the polymer are cleaved due to the action of chymotrypsin or trypsin, primary amines are formed, These primary amines as well as those of the lysine residues were quantified by the ninhydrin assay[81]. Upon incubation of poly(N_3-Phe-Ala-Lys-propargyl amide) with chymotrypsin (0.4 μM) at pH 7.5 an increase of primary amines was observed, until a plateau value was obtained after about 70 h (Figure 8A). The final amine concentration was approx. 1.7 times the initial concentration of primary amines, indicating that the majority of the repeating units were cleaved by chymotrypsin.

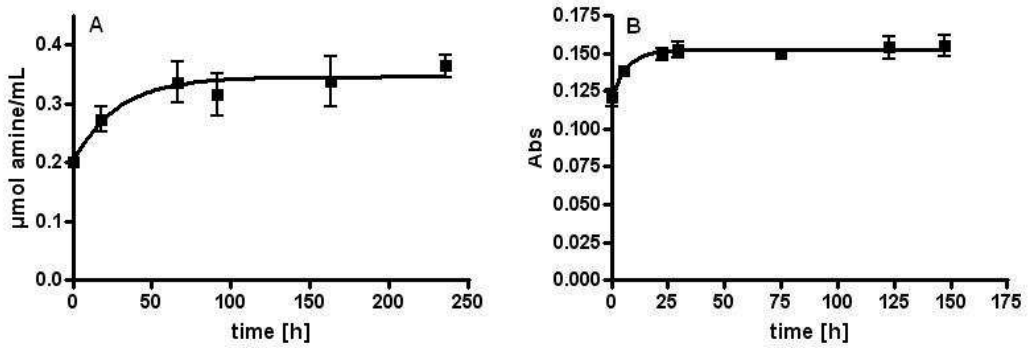


Figure 8: Time courses of the concentration of primary amines in chymotrypsin-incubated samples of poly(N_3-Phe-Ala-Lys-propargyl amide) (entry 5) with an enzyme concentration of 0.4 µM (A) and poly(N_3-Phe-Ala-Glyc-Lys-propargyl amide) (entry 10) with an enzyme concentration of 4 µM (B) as determined by the ninhydrin assay (average ± SD of three experiments).

Figure 9 shows mass spectra of poly(N_3-Phe-Ala-Lys-propargyl amide) degraded with chymotrypsin at pH 7.5 after t = 0, 91 and 235 h. Shortly after addition of the enzyme, only a small part of the polymer chains have been cleaved by chymotrypsin. After 91 h only oligomers were visible and after 238 h only small fragments remain, indicating that this polymer is sensitive toward digestion by chymotrypsin. The degradation of poly(N_3-Phe-Ala-Glyc-Lys-propargyl amide) with chymotrypsin was conducted at pH 5.0 with the same enzyme concentration (0.4 µM) as with the degradation of poly(N_3-Phe-Ala-Lys-propargyl amide). However after 240 hours hardly any degradation was observed. Therefore the experiment was repeated with 10 times (4 µM) the initial enzyme concentration. According to the ninhydrine assay, after 30 hours a plateau value was obtained, which corresponds with about a third of the scissile bonds cleaved (Figure 8B). The degradation of the polymer was unexpectedly slow compared to the degradation rate of the monomer. Nevertheless, it was concluded that both polymers are sensitive towards enzymatic hydrolysis by chymotrypsin.

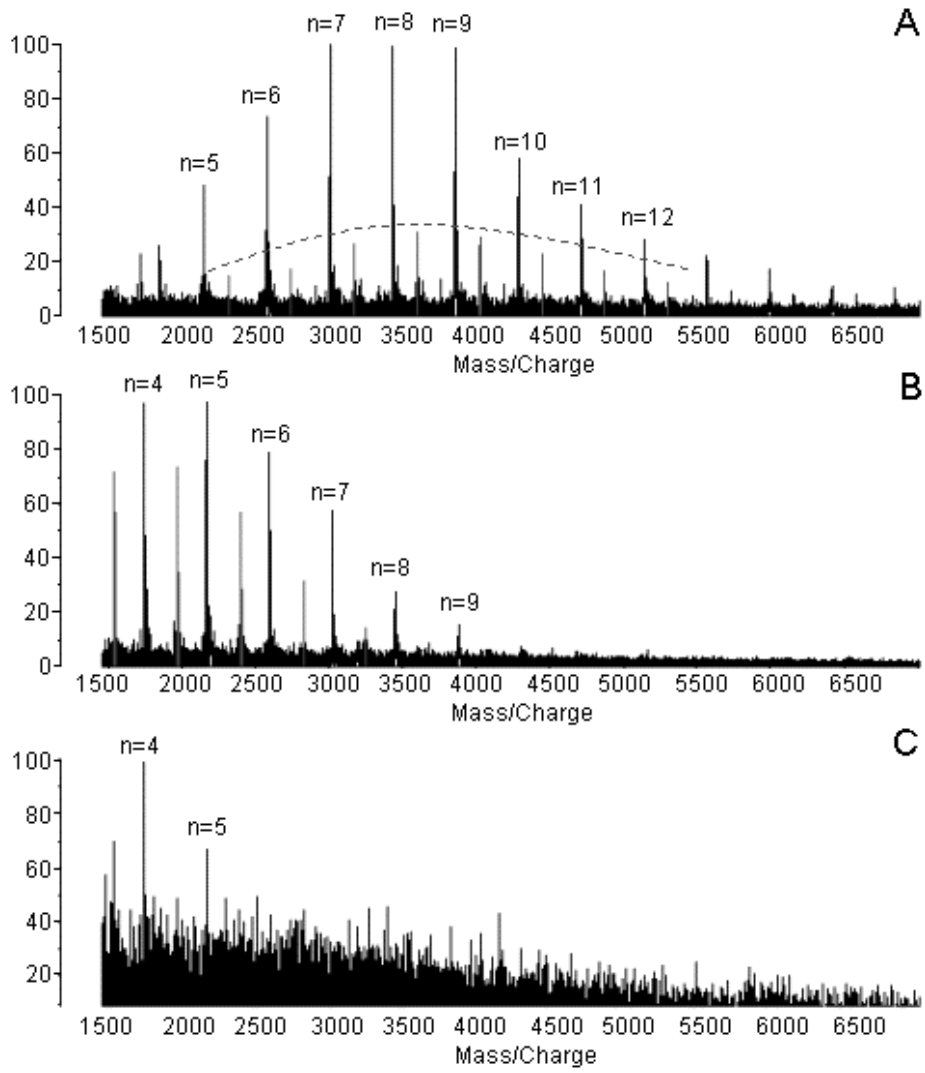


Figure 9: Mass spectra of poly(N_3-Phe-Ala-Lys-propargyl amide) (entry 5) incubated with chymotrypsin after 0 h (A), 91 h (B) and 235 h (C). Gray bars are cleaved polymer products, also indicated by the dotted line.

Trypsin induced degradation of poly(N_3-Phe-Ala-Lys-propargyl amide) and poly(N_3-Phe-Ala-Glyc-Lys-propargyl amide). From Figure 10B it can been seen that poly(N_3-Phe-Ala-Glyc-Lys-propargyl amide) was degraded in the presence of trypsin (0.8 μM) at pH 5.0 and after 30 h a plateau value in amine concentration was obtained of about 1.8 times the initial amine concentration. When the other polymer, poly(N_3-Phe-Ala-Lys-propargyl amide), was incubated with the same concentration of trypsin at pH 7.5, the polymer was almost instantaneously degraded (results not shown), which is in agreement with the short $t_{1/2}$ of the monomer. Therefore, the polymer was degraded with a lower trypsin concentration (0.016 μM). At this trypsin concentration the polymer

was completely degraded within 50 h, as evidenced by the fact that the concentration of primary amines was doubled (Figure 10A).

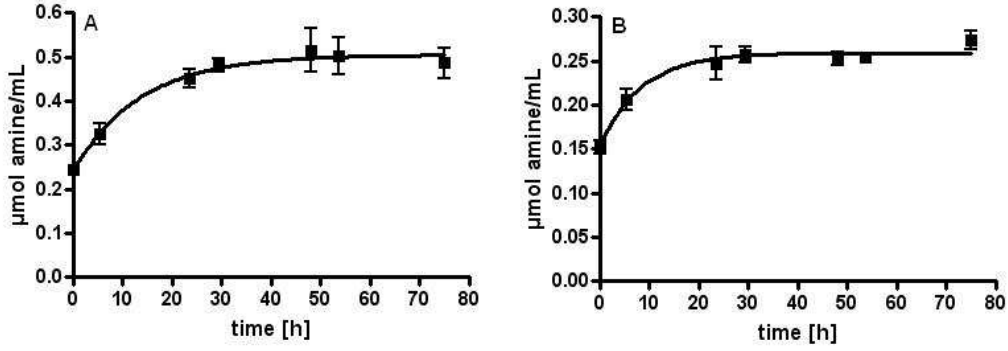


Figure 10: Time courses of the concentration of primary amines in trypsin-incubated samples of poly(N_3-Phe-Ala-Lys-propargyl amide) (0.016 µM) (entry 5) (A) and poly(N_3-Phe-Ala-Glyc-Lys-propargyl amide) (0.8 µM) (entry 10) (B) as determined by the ninhydrin assay (average ± SD of three experiments).

This was also confirmed by HPLC analysis, which showed only a single major degradation product in the reaction mixture after 80 h (Figure 11). This fragment was identified by LC/MS/MS and corresponded with the expected degradation product as represented by its structural formula in Figure 11B. These results showed that not only degradable polymers were synthesized, but also that we were able to control and identify the chemical nature of the degradation products.

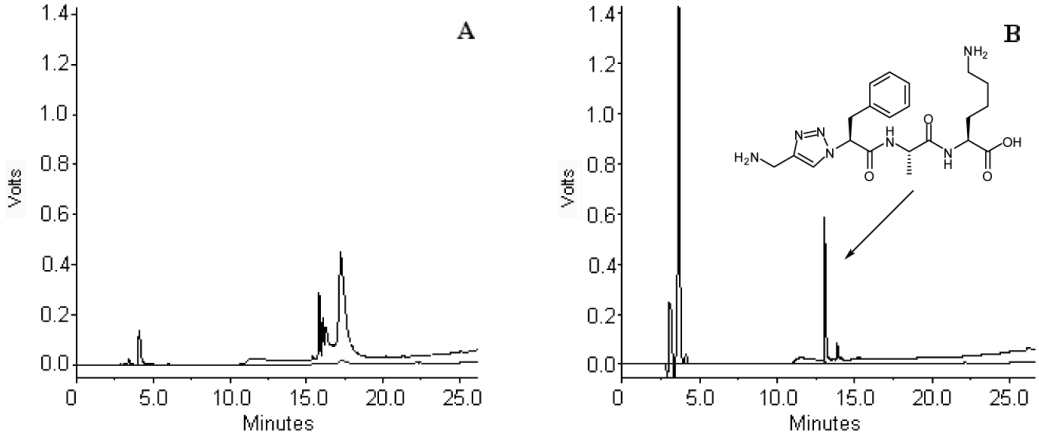


Figure 11: HPLC chromatograms of poly(N_3-Phe-Ala-Lys-propargyl amide) (entry 5) before (A) and after (B) incubation with trypsin (0.016 µM) for 70 h at pH 7.5 and 37 °C. The structural identity of the degradation products was confirmed by LC/MS/MS.

4.4 Conclusions

It has been demonstrated that the microwave-assisted copper(I)-catalyzed 1,3-dipolar cycloaddition reaction can be successfully employed for the synthesis of polymers with repeating peptide sequences starting from tri- and tetrapeptides as monomer containing an unprotected functional amino group. The molecular weight of the polymers could be tailored between 4,500 and 14,000 Da (33 to 100 amino acid residues) depending on the monomer concentration used during the polymerization.
The synthesized polymers were sensitive for the proteases chymotrypsin and trypsin. When polymers derived from monomer **1** (N_3-Phe-Ala-Lys-propargyl amide) were degraded by trypsin, a single and well-defined degradation product was formed and identified by LC/MS/MS. Polymers derived from monomer **2** (N_3-Phe-Ala-Glyc-Lys-propargyl amide) were also sensitive to chemical hydrolysis with a good control over the degradation products as well, as confirmed by MALDI-TOF analysis.
To summarize, the microwave-assisted copper(I)-catalyzed 1,3-dipolar cycloaddition reaction is an attractive approach towards the synthesis of novel designed biomedical polymers and materials.

4.5 Experimental Part

General procedures. Chemicals were obtained from commercial sources and used without further purification. Reactions were carried out at room temperature unless stated otherwise. Column chromatography was performed with Silica-P Flash silica gel (Silicycle). Thin layer chromatography (TLC) was performed on Merck silica gel 60 F-254 plates. Spots were visualized by UV light, ninhydrin, TDM/Cl_2,[82] $KMnO_4$ or triphenylphosphine/ninhydrin.[83] Microwave reactions were carried out in a Biotage initiator apparatus. ^{1}H-NMR spectra were recorded on a Varian Mercury plus 300 MHz and chemical shift values (δ) are given in ppm relative to TMS. ^{13}C-NMR spectra (75 MHz) were recorded using the attached proton test (APT) pulse sequence[81] and chemical shift values are given in ppm relative to $CDCl_3$ (77.0 ppm) or DMSO-d_6 (39.5 ppm). Fourier transform infrared spectra (FTIR) were measured on a Bio-Rad FTS-25 spectrophotometer. Thermogravimetric analysis (TGA) was carried out on a TGA 51 Thermogravimetric analyzer (TA Instruments). MALDI-TOF analyses were performed on a Kratos Axima CFR apparatus, with ACTH(18-39) as an external reference (monoisotopic $[M + H]^+$ 2465.1989) and α-cyano-4-hydroxycinnamic acid as matrix.

Gel permeation chromatography. GPC was performed on a Waters 2695 Controller equipped with a refractive index detector (model 2414). The analyses were run on a PLgel MIXED-D column (particle size: 5 μm) (Polymer Laboratories) at 40 °C using 10 mM LiCl in DMF as the mobile phase

at a flow rate of 0.7 mL/min. The polymers were dissolved overnight in DMF (containing 10 mM LiCl) at a concentration of 5 mg/mL and filtered through a 0.45 μm filter prior analysis. The samples were analyzed and calibrated using PEG as standards (M: 194-439,600 g/mol; Polymer Laboratories). Peak areas were determined with Empower Software Version 1154 (waters Associates inc.).

Differential scanning calorimetry. DSC was carried out on a Q1000 differential scanning calorimeter (TA Instruments). For the DSC measurement, the samples were heated (10 °C/min) from room temperature to 150 °C. Then, these samples were cooled to -10 °C and subsequently heated (10 °C/min) for a second time to 150 °C.

HPLC. Analytical RP-HPLC runs were carried out on a Shimadzu automated HPLC system equipped with a UV/VIS detector operating at λ = 214 and 254 nm and using an Alltech Prosphere C18 column (250 × 4.6 mm, particle size: 5 μm, pore size: 300Å) at a flow rate of 1 mL/min using a linear gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95:5 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 20 or 40 min. LC/MS(MS) runs were performed on a Finnigan LCQ Deca XP MAX LC/MS equipped with a Shimadzu 10A VP analytical HPLC system. The samples were dissolved in 10% formic acid in CH_3CN/H_2O 1:1 v/v and analyzed on a Phenomenex Gemini C18 column (150 × 4.6 mm, particle size: 3 μm, pore size: 110Å) at a flow rate of 1.0 mL/min using a linear gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95:5 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 50 min.

*4.5.1 Synthesis of azido-phenylalanyl-alanyl-lysyl-propargyl amide (**1**)*

H-Lys(Boc)-propargyl amide (3). Fmoc-Lys(Boc)-OH (1.0 g, 2.1 mmol), BOP (1.04 g, 2.4 mmol), DIPEA (1.1 mL, 6.4 mmol) and propargylamine hydrochloride (0.21 g, 2.2 mmol) were dissolved in DCM (30 mL). After stirring for 16 h, the solvent was removed in vacuo and the residue was redissolved in EtOAc (100 mL). The solution was washed with 1N $KHSO_4$ (3×), H_2O (1×), 5% $NaHCO_3$ (3×) and brine (3×). The organic layer was dried (Na_2SO_4), filtered and concentrated in vacuo. The obtained crude Fmoc-Lys(Boc)-propargyl amide was used without further purification in the next step. Yield: 95% (1.02 g) as a white solid. R_f = 0.67 (DCM/MeOH 9:1 v/v). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.36-1.49 (m, 4H, H^γ Lys (2H) and H^δ Lys (2H)), 1.43 (m, 9H, CH_3 Boc), 1.64-1.87 (m, 2H, H^β Lys), 2.14 (s, 1H, C≡CH), 3.10 (m, 2H, H^ε Lys), 4.00 (m, 2H, $C\underline{H}_2C{\equiv}CH$), 4.19 (m, 2H, H^α Lys (1H) and CH Fmoc (1H)), 4.41 (m, 2H, CH_2 Fmoc), 4.66 (broad s, 1H, NH urethane), 5.62 (m, 1H, NH amide), 6.73 (broad s, 1H, NH urethane), 7.16-7.77 (m, 8H, arom H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 22.6, 28.6, 29.4, 29.8, 32.2, 40.0, 47.3, 54.8, 67.3, 71.9, 79.4, 120.2, 125.3, 127.3, 128.0, 129.2, 141.5, 143.9, 156.4, 171.7.

Fmoc-Lys(Boc)-propargyl amide (0.5 g, 1 mmol) was dissolved in 20% piperidine in THF (20 mL). After stirring for 1 h, the solvent was removed in vacuo and the residue was coevaporated with toluene (3×) and chloroform (3×). The residue was purified by column chromatography with (DCM/MeOH 9:1 v/v) as eluent. H-Lys(Boc)-propargyl amide was obtained as yellow oil in 81% yield (0.23 g) . R_f = 0.27 (DCM/MeOH 9:1 v/v). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.37-1.87 (m, 6H, H^γ Lys (2H), H^δ Lys (2H) and H^β Lys (2H)), 1.44 (s, 9H, CH_3 Boc), 2.23 (s, 1H, C≡CH), 3.11 (m, 2H, H^ε Lys), 3.67 (m, 1H, H^α Lys), 4.04 (m, 2H, C$\underline{H_2}$C≡CH), 4.62 (broad s, 1H, NH urethane), 7.58 (broad s, 1H, NH amide).

H-Ala-Lys(Boc)-propargyl amide (4). Fmoc-Ala-OH (4.5 g, 14.5 mmol), BOP (6.4 g, 14.3 mmol) and H-Lys(Boc)-propargyl amide (3.8 g, 13.4 mmol) were dissolved in DCM/EtOAc 25:1 v/v (520 mL) followed by the addition of DIPEA (4.6 mL, 27.8 mmol). After stirring for 16 h, the solvent was removed in vacuo and the residue was redissolved in EtOAc. The solution was washed with 1N $KHSO_4$ (3×), H_2O (1×), 5 % $NaHCO_3$ (3×), H_2O (1×) and brine (3×). During the washing steps the formed precipitate was collected by filtration. The organic layer was dried (Na_2SO_4), filtered and evaporated in vacuo. The residue was combined with the precipitated product and purified by column chromatography (DCM/MeOH 9:1 v/v). Fmoc-Ala-Lys(Boc)-propargyl amide was obtained as a white powder in quantitative yield (8.3 g). R_f = 0.50 (DCM/MeOH 9:1 v/v). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.26-1.66 (m, 9H, H^β Lys (2H), H^γ Lys (2H), H^δ Lys (2H) and H^β Ala (3H)), 1.42 (s, 9H, CH_3 Boc), 2.19 (s, 1H, C≡CH), 3.06 (m, 2H, H^ε Lys), 4.00 (m, 2H, C$\underline{H_2}$C≡CH), 4.22 (m, 2H, H^α Lys (1H) and CH Fmoc (1H)), 4.33-4.44 (m, 3H, H^α Ala (1H) and CH_2 Fmoc (2H)), 4.88 (broad s, 1H, NH amide), 5.90 (broad s, 1H, NH urethane), 7.24-7.78 (m, 8H, arom H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 18.3, 22.5, 28.3, 28.9, 31.3, 47,1, 49,6, 50.7, 52.6, 67.0, 71.3, 79.3, 119.9, 125.0, 127.1, 127.8, 141.3, 143.7, 156.4, 171.2, 172.8.

Fmoc-Ala-Lys(Boc)-propargyl amide (8.3 g, 14 mmol) was dissolved in 20% piperidine in THF (150 mL) and the reaction mixture was stirred for 75 min at room temperature. Then, the solvent was evaporated under reduced pressure and the residue was coevaporated with toluene (3×) and chloroform (3×) and subsequently purified by column chromatography (DCM/MeOH 98:2 v/v). H-Ala-Lys(Boc)-propargyl amide was obtained as a white powder in 84% yield (4.0 g) R_f = 0.15 (DCM/MeOH 95:5 v/v). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.25-1.92 (m, 6H, H^β Lys (2H), H^γ Lys (2H) and H^δ Lys (2H)) 1.35 (d (*J* 6.9 Hz), 3H, H^β Ala), 1.44 (s, 9H, CH_3 Boc), 2.22 (s, 1H, C≡CH), 3.10 (m, 2H, H^ε Lys), 3.52 (m, 1H, H^α Ala), 4.02 (s, 2H, C$\underline{H_2}$C≡CH), 4.40 (m, 1H, H^α Lys), 4.67 (broad s, 1H, NH urethane), 6.96 (broad s, 1H, NH amide), 7.27 (broad s, 2H, NH_2), 7.80 (broad d, 1H, NH Lys). ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 21.4, 22.4, 27.8, 28.2, 29.1, 32.2, 50.3, 51.7, 73.0, 77.3, 80.9, 155.5, 171.4, 175.5.

N_3-Phe-OSu (5). N_3-Phe-OH was synthesized according to a procedure described by Lundquist et al. [84,85] in 95% yield. A detailed procedure for the synthesis of azidophenylalanine is described in chapter 3. R_f = 0.80 (DCM/MeOH/AcOH 95:5:1 v/v/v). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 3.01-3.08 (dd (J_{ax} 8.9 Hz, J_{ab} 14.2 Hz), 1H, H^{β}), 3.21-3.28 (dd (J_{ax} 5.0 Hz, J_{ab} 14.0 Hz), 1H, H^{β}), 4.14 (m, 1H, αCH), 7.25-7.38 (m, 5H, arom H), 10.72 (broad s, 1H, OH); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: 37.4, 63.0, 127.4, 128.7, 129.2, 135.5, 175.9.

A solution of N_3-Phe-OH (4.2 g, 22.2 mmol) in DCM (250 mL) was cooled on ice and N-hydroxysuccinimide (2.8 g, 24.7 mmol) followed by DCC (4.83 g, 23.5 mmol) were added and the obtained reaction mixture was stirred overnight at room temperature. Subsequently, the reaction mixture was filtered over celite and the solvent was removed in vacuo. The residue was coevaporated with chloroform (2×) and dissolved in CH_3CN and this solution was filtered again over celite. The solvent was removed in vacuo and the residue was coevaporated with chloroform (3×). The residue was purified by column chromatography (DCM/MeOH 97:3 v/v) and pure N_3-Phe-OSu was obtained as a white solid in 67% yield (2.9 g). R_f = 0.75 (DCM/MeOH/AcOH 95:5:1 v/v/v). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 2.88 (s, 4H, CH_2 OSu), 3.12-3.20 (dd (J_{ax} 9.1 Hz, J_{ab} 14.3 Hz), 1H, H^{β} Phe), 3.33-3.40 (dd (J_{ax} 5.0 Hz, J_{ab} 14.2 Hz), 1H, H^{β} Phe), 4.41 (m, 1H, H^{α}), 7.26-7.39 (m, 5H, arom H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 26.0, 38.2, 61.6, 128.1, 129.3, 129.7, 135.1, 166.1, 168.8.

N_3-Phe-Ala-Lys(Boc)-propargyl amide (6). H-Ala-Lys(Boc)-propargyl amide **(4)** (3.5 g, 10 mmol) and N_3-Phe-OSu (2.9 g, 10 mmol) were dissolved in DMF (100 mL). To this solution Et_3N (2.6 mL, 18.7 mmol) was added and the reaction mixture was stirred for 16 h. Then, the solvent was removed under reduced pressure and residue was redissolved in EtOAc and the obtained solution was washed with 1N $KHSO_4$ (3×), H_2O (1×), 5 % $NaHCO_3$ (3×), H_2O (1×) and brine (3×). The organic layer was dried (Na_2SO_4), filtered and concentrated in vacuo. The crude product was purified by column chromatography (EtOAc/hexane 2:1 v/v) to give compound **6** as a white solid in 50% yield (2.6 g). R_f = 0.31 (EtOAc/hexane 2:1 v/v). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 1.22-1.51 (m, 4H, H^{γ} Lys (2H) and H^{δ} Lys (2H)), 1.28 (d (*J* 7.2 Hz), 3H, H^{β} Ala), 1.43 (s, 9H, CH_3 Boc) 1.61-1.89 (m, 2H, H^{β} Lys), 2.23 (t (*J* 2.5 Hz), 1H, C≡C*H*), 3.02-3.11 (m, 3H, H^{ε} Lys (2H) and H^{β} Phe (1H)), 3.27-3.33 (dd (J_{ax} 4.4 Hz, J_{ab} 14.0 Hz), 1H, H^{β} Phe)**,** 4.01 (m, 2H, C$\underline{H}_2$C≡CH), 4.25 (m, 1H, H^{α} Phe), 4.37-4.48 (m, 2H, H^{α} Ala and H^{α} Lys), 4.81 (broad t, 1H, NH urethane), 7.06 (m, 3H, NH amide, NH amide Lys, NH amide Ala), 7.24-7.34 (m, 5H, arom H). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: 18.3, 22.6, 27.9, 28.2, 29.2, 31.8, 36.9, 48.2, 52.3, 62.2, 73.0, 77.3, 80.9, 126.6, 128.4, 129.0, 136.9, 155.5, 168.7, 171.2, 171.5.

N_3-Phe-Ala-Lys-propargyl amide (1). N_3-Phe-Ala-Lys(Boc)-propargyl amide **(6)** (2.6 g, 5 mmol) was dissolved in TFA/DCM 1:1 v/v (100 mL) and the solution was stirred for 75 min at room temperature. Subsequently, the solvent was evaporated in vacuo and the crude product was coevaporated with toluene (3×) and chloroform (3×). The crude product was dissolved in water and the aqueous phase was washed with Et_2O. The water layer was lyophilized to yield quantitatively monomer **1** as a white solid (2.7 g). R_f = 0.31 (EtOAc/hexane 2:1 v/v). ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 1.22-1.36 (m, 2H, H^{γ} Lys), 1.27 (d (*J* 7.2 Hz), 3H H^{β} Ala), 1.48-1.66 (m, 4H, H^{β} Lys (2H) and H^{δ} Lys (2H)), 2.75 (m, 2H, H^{ε} Lys), 2.85-3.14 (m, 3H, H^{β} Phe (2H) and C≡CH (1H)), 3.86 (m, 2H, C$\underline{H_2}$C≡CH), 4.09 (m, 1H, H^{α} Phe), 4.23 (m, 1H, H^{α} Lys), 4.34 (m, 1H, H^{α} Ala), 7.16-7.35 (m, 5H, arom H), 7.73 (broad s, 2H, NH_2), 8.09 (broad d (*J* 8.0 Hz), 1H, NH amide Lys), 8.37 (broad t (*J*5.5 Hz), 1H, NH amide), 8.46 (broad d (*J* 7.2 Hz), 1H, NH amide Ala). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 18.2, 22.2, 26.6, 27.9, 31.4, 36.9, 38.7, 48.4, 52.1, 62.2, 73.1, 81.0, 126.7, 128.4, 129.1, 137.0, 168.8, 171.2, 171.7. ESI-MS calcd for $C_{21}H_{29}N_7O_3$: 427.23 found m/z [$M+H^+$] = 428.90.

4.5.2 Synthesis of azido-phenylalanyl-alanyl-glycoloyl-lysyl-propargyl amide (2)

HO-Glyc-OBzl (7). HO-Glyc-OBzl was synthesized according to the procedure described by Shin.[86] In short, glycolic acid (1.0 g, 13.5 mmol) was dissolved in MeOH/H_2O 20:3 v/v (23 mL) and a solution of Cs_2CO_3 (2 g) in H_2O (8 mL) was added until pH 7 was reached. The solvent was removed in vacuo and the residue was dissolved in DMF (50 mL). Benzyl bromide (1.65 mL, 13.5 mmol) was added drop-wise and the obtained mixture was stirred for 16 h. The reaction mixture was quenched by the addition of brine (50 mL). The aqueous layer was extracted with EtOAc (3×). The combined EtOAc layers were washed with H_2O (1×) and brine (1×) and subsequently dried ($MgSO_4$), filtered and concentrated in vacuo. The residue was purified by column chromatography with hexane/EtOAc (4:1 v/v). HO-Glyc-OBzl was obtained as a yellow oil in 73% yield (1.64 g). R_f = 0.27 (DCM/MeOH 9:1 v/v). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 2.64 (broad s, 1H, OH), 4.19 (s, 2H, CH_2 Glyc), 5.22 (s, 2H, CH_2 Bzl), 7.35 (m, 5H, arom H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 60.6, 67.2, 128.4, 128.6, 135.0, 173.2.

Boc-Ala-Glyc-OH (8). Boc-Ala-OH (7 g, 37 mmol) and HO-Glyc-OBzl **(7)** (6.15 g, 37 mmol) were dissolved in DCM (400 mL) and the solution was cooled on ice. DCC (8.29 g, 37 mmol) and 4-dimethylaminopyridine (DMAP) (0.45 g, 0.37 mmol) were added and the reaction mixture was stirred overnight at room temperature. The reaction mixture was filtered over celite and the solvent was removed in vacuo. The residue was redissolved in EtOAc and the solution was subsequently washed with 1N $KHSO_4$ (3×), H_2O (1×) and brine (3×), dried (Na_2SO_4), filtered and concentrated in vacuo. The residue was purified by column chromatography (hexane/EtOAc 4:1 v/v). Boc-Ala-Glyc-OBzl was obtained as a yellow oil in 72% yield (9.0 g). R_f = 0.28 (hexane/EtOAc 4:1 v/v). ^{1}H NMR

($CDCl_3$, 300 MHz) δ: 1.41 – 1.44 (m, 9H, H^{β} Ala (3H) and CH_3 Boc), 4.41 (m, 1H, H^{α} Ala), 4.57 – 4.85 (dd (J_{ax} 16.6 Hz, J_{ab} 67.9 Hz), 2H, CH_2 Glyc), 5.04 (m, 1H, NH urethane), 5.19 (s, 2H, CH_2 Bzl), 7.26 - 7.40 (m, 5H, arom H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 18.3, 28.8, 49.0, 60.9, 67.1, 79.9, 128.4, 128.6, 134.9, 167.2, 172.8.

Boc-Ala-Glyc-OBzl (9 g, 26.7 mmol) was dissolved in THF (300 mL) and H_2O (200 mL) and Pd/C (0.46 g) was added. The reaction vessel was purged with H_2 (3×) and placed under an atmosphere of H_2. After 16 h the reaction mixture was filtered over celite and the filtrate was evaporated and the residue was coevaporated with toluene (3×) and chloroform (3×). Boc-Ala-Glyc-OH **(8)** was obtained as a yellow oil in 94% yield (6.2 g). R_f = 0.25 ($CHCl_3$/MeOH/AcOH 95:20:3 v/v/v). ^1H NMR ($CDCl_3$, 300 MHz) δ: 1.23-1.44 (m, 12H, H^{β} Ala (3H) and CH_3 Boc (9H)), 4.25 – 4.79 (m, 3H, H^{α} Ala (1H) and CH_2 Glyc (2H)), 5.16 (broad d, 1H, NH urethane), 9.85 (broad s, 1H, COOH).

N_3-Phe-Ala-Glyc-OH (9). Boc-Ala-Glyc-OH **(8)** (4.46 g, 18 mmol) was dissolved in TFA/DCM 1:1 v/v (80 mL). After 1 h of stirring, the solvent was evaporated in vacuo and the product was coevaporated with toluene (3×) and chloroform (3×). The oily residue was dissolved in DMF (100 mL) and N_3-Phe-OSu **(5)** (7.56 g, 18 mmol) followed by TEA (11 mL, 54 mmol) were added. The obtained reaction mixture was stirred for 16 h. The solvent was removed in vacuo and the compound was dissolved in EtOAc and washed with 1N $KHSO_4$ (3×), H_2O (1×) and brine (3×). The organic layer was dried (Na_2SO_4), filtered and concentrated in vacuo. The crude product was purified by column chromatography (hexane/EtOAc/AcOH 70:30:1 v/v/v). Compound **9** was obtained as a white solid in 40% yield (2.3 g). R_f = 0.6 ($CHCl_3$/MeOH/AcOH 95:20:3 v/v/v). ^1H NMR ($CDCl_3$, 300 MHz) δ: 1.37 (d (J 7.4 Hz), 3H, H^{β} Ala), 3.01-3.08 (dd (J_{ax} 8.0 Hz, J_{ab} 14.0 Hz), 1H, H^{β} Phe), 3.29-3.35 (dd (J_{ax} 4.4 Hz, J_{ab} 14.0 Hz), 1H, H^{β} Phe), 4.30 (m, 1H, H^{α} Phe), 4.57 – 4.79 (m, 3H, CH_2 Glyc (2H) and H^{α} Ala (1H)), 6.83 (broad d (J 7.4 Hz), 1H, NH amide Ala), 7.22 – 7.39 (m, 5H, arom H), 9.88 (Broad s, 1H, COOH). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 17.8, 38.4, 47.9, 60.9, 64.9, 127.3, 128.6, 129.5, 135.6, 168.9, 171.1, 171.7.

N_3-Phe-Ala-Glyc-Lys-propargyl amide (2). To a solution of H-Lys(Boc)-propargyl amide **(3)** (1.8 g, 6.3 mmol) in DCM (100 mL), N_3-Phe-Ala-Glyc-OH **(9)** (2 g, 6.3 mmol), DIPEA (2.7 mL, 15.7 mmol) and BOP (2.2 g, 6.9 mmol) were added and the reaction mixture was stirred for 16 h. The solvent was removed in vacuo and the product was redissolved in EtOAc (100 mL) and this solution was washed with 1N $KHSO_4$ (3×), H_2O (1×), 5 % $NaHCO_3$ (3×), H_2O (1×) and brine (3×). The organic layer was dried (Na_2SO_4), filtered and evaporated in vacuo. The crude product was purified by column chromatography (DCM/MeOH 98:2 v/v). N_3-Phe-Ala-Glyc-Lys(Boc)-propargyl amide was obtained as an off-white solid in 57% yield (2.1 g). R_f = 0.63 (DCM/MeOH 9:1 v/v). ^1H NMR ($CDCl_3$, 300 MHz) δ: 1.34 – 1.54 (m, 7H, H^{β} Ala (3H), H^{γ} Lys (2H) and H^{δ} Lys (2H)), 1.43 (s, 9H,

CH_3 Boc), 1.76-1.93 (m, 2H, H^{β} Lys), 2.22 (s, 1H, C≡CH), 3.02 – 3.13 (m, 3H, H^{ε} Lys (2H) and H^{β} Phe (1H)), 3.30-3.36 (dd (J_{ax} 4.1 Hz, J_{ab} 14.0 Hz), 1H, H^{β} Phe), 4.03 (m, 2H, C$\underline{H}_2$C≡CH), 4.35 – 4.57 (m, 3H, H^{α} Phe (1H), H^{α} Lys (1H) and H^{α} Ala (1H)), 4.62 – 4.74 (m, 3H, CH_2 Glyc (2H) and NH urethane), 6.77 (broad s, 2H, NH amide (1H) and NH amide Lys (1H)), 7.20 – 7.35 (m, 5H, arom Phe). ^{13}C NMR ($CDCl_3$/CD_3OD, 75 MHz) δ: 17.1, 22.6, 28.3, 28.4, 29.1, 29.4, 31.5, 38.3, 40.0, 48.6, 52.8, 63.3, 64.7, 71.7, 79.1, 127.3, 128.6, 149.4, 135.7, 156.1, 169.3, 171.4.

N_3-Phe-Ala-Glyc-Lys(Boc)-propargyl amide (2 g, 3.4 mmol) was dissolved in TFA/DCM 1:1 v/v (80 mL) and after stirring for 1 h at room temperature the solvent was evaporated in vacuo and the crude product was subsequently coevaporated with toluene (3×) and chloroform (3×), dissolved in H_2O /CH_3CN 1:1 v/v and lyophilized. Compound **2** was obtained as a white solid in 96% yield (1.96 g). R_f = 0.24 ($CHCl_3$/MeOH/AcOH 95:20:3 v/v/v). ^{1}H NMR ($CDCl_3$/CD_3OD, 300 MHz) δ: 1.26-1.46 (m, 2H, H^{γ} Lys), 1.42 (d (*J* 7.2 Hz), 2H, H^{β} Ala), 1.67-1.87 (m, 4H, H^{β} Lys (2H) and H^{δ} Lys (2H)), 2.25 (s, 1H, C≡CH), 2.84 – 2.97 (m, 2H, H^{ε} Lys), 2.99-3.06 (dd (J_{ax} 8.3 Hz, J_{ab} 14.0 Hz), 1H, H^{β} Phe), 3.24-3.31 (dd (J_{ax} 4.7 Hz, J_{ab} 14.0 Hz), 1H, H^{β} Phe), 4.00 (m, 2H, C$\underline{H}_2$C≡CH), 4.22 (m, 1H, H^{α} Phe), 4.41 – 4.74 (m, 4H, H^{α} Lys (1H), H^{α} Ala (1H) and CH_2 Glyc (2H)), 7.24-7.35 (m, 5H, arom H), 7.48 (broad d (*J* 6.9 Hz), 1H, NH amide Ala), 7.68 (broad d (*J* 8.3 Hz), 1H, NH amide Lys), 7.94 (broad t (*J* 5.2 Hz), 1H, NH amide). ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 16.8, 22.2, 26.6, 28.0, 31.4, 36.9, 47.7, 51.9, 62.2, 62.4, 73.0, 81.0, 126.7, 128.4, 129.1, 136.8, 166.3, 169.3, 171.0, 171.6. ESI-MS calcd for $C_{23}H_{31}N_7O_5$: 485.24 found m/z [$M+H^+$] = 486.99.

Synthesis of acetylene-terminated PEG 2000 monomethylether. PEG monomethylether Mw 2000 (20 g, 10 mmol) was dissolved in dry THF (300 mL). NaH (1 g of a 60% suspension in mineral oil, 25 mmol) and a solution of propargyl bromide in toluene (1.45 mL of a 80% solution in toluene, 2 g, 13 mmol) were added and the reaction mixture was stirred for 16 h. Subsequently the solvent was removed in vacuo and the residue was purified by column chromatography (DCM/MeOH 95:5 v/v). Acetylene-terminated PEG 2000 monomethylether was obtained as a white solid in 85% yield (17.0 g). R_f = 0.54 (DCM/MeOH/AcOH 95:5:1 v/v/v). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 2.46 (s, C≡CH), 3.38 (s, CH_3), 3.66 (m, CH_2 (PEG)), 4.20 (d, C$\underline{H}_2$C≡CH), ^{13}C NMR (75 MHz, $CDCl_3$) δ: 58.2, 58.8, 68.9, 70.4, 71.8, 74.4.

4.5.3 Polymer Synthesis

An overview of all polymerization reactions is given in table 1. In a typical experiment (entry 4) 200 mg (0.4 mmol) monomer **1** was dissolved in 1 mL degassed DMF and CuOAc (2.5 mg, 0.02 mmol) was added. The reaction mixture was placed in the microwave reactor (Biotage) and irradiated at 100 °C for 30 min. During irradiation, the clear solution was transformed into a turbid gel. The gel was

dissolved in an additional amount of DMF (3 mL) and the solvent was removed in vacuo. The obtained solid was dissolved in H_2O/CH_3CN 1:1 v/v and lyophilized to yield quantitatively the polymer as a greenish solid.

As a typical example for the characterization of poly(N_3-Phe-Ala-Lys-propargyl amide), polymer entry 5 was used. ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 1.20-1.30 (m, 5H, H^β Ala (3H), H^β Lys (2H)), 1.53 (m, 4H, H^γ Lys (2H) and H^δ Lys (2H)), 2.73-2.89 (m, 4H, H^β Phe (2H), H^ε Lys (2H)) , 4.26 (m, 4H, CONHC<u>H</u>$_2$ (2H), H^α Lys (1H) and H^α Ala (1H)), 5.70 (m, 1H, H^α Phe), 7.17 (m, 5H, arom H), 7.75 (broad s, 2H, NH_2), 7.93 (m, 1H, CH triazole), 8.09 (broad s, 1H, NH amide Lys), 8.40 (broad s, 1H, NH amide), (broad d, 1H, NH amide Ala).

As a typical example for the characterization of poly(N_3-Phe-Ala-Glyc-Lys-propargyl amide), polymer entry 10 was used. ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 1.10-1.80 (m, 9H, H^β Ala (3H), H^β Lys (2H), H^γ Lys (2H) and H^δ Lys (2H)), 2.73 (m, 2H, H^ε Lys), 4.00-4.63 (m, 6H, CONHC<u>H</u>$_2$ (2H), CH_2 Glyc (2H), H^α Ala (1H), H^α Lys (1H)), 5.63 (m, 1H, H^α Phe), 7.14 – 7.28 (m, 5H, arom H), 7.62 – 9.11 (m, 4H, NH amide Lys (1H), NH amide Ala (1H), NH amide (1H), CH triazole (1H)). DSC T_g: 129 °C.

4.5.4 Monomer Degradation

Chemical hydrolysis: Buffers used were Na-acetate at pH 5.0 and 5.5, Na-phosphate at pH 6.5, 7.4 and 8.0 and HEPES-HCl at pH 8.5, all at a concentration of 100 mM. N_3-Phe-Ala-Glyc-Lys-propargyl amide **(2)** (9 mg) was dissolved in buffer (3 mL) and incubated at 37 ˚C in a thermostated water bath. The pH was measured before and after degradation at 37 ˚C. At regular time points a sample (100 μL) was drawn and quenched with ice-cold 1 M Na-acetate buffer pH 3.8 (200 μL) and stored at 4˚C prior to HPLC analysis.

Enzymatic degradation. Buffers used were Tris buffer pH 7.5 for monomer **1** and Na-acetate pH 5.0 for monomer **2**. In a typical experiment, 3 mg of monomer was dissolved in 1 mL 100 mM buffer and either 40 μL α-chymotrypsin (type II from bovine pancreas) dissolved in 1 mM HCl (0.5 mg/mL) and 40 μL 2M $CaCl_2$, or 40 μL trypsin (Type IX-S from porcine pancreas) dissolved in 1 mM HCl (0.01 mg/mL for monomer **1** or 0.5 mg/mL for monomer **2**) was added (table 3). The samples were incubated at 37˚C in a thermostated water bath. At different time points 100 μL of sample was drawn and quenched with 200 μL ice-cold 1M Na-acetate buffer pH 3.8. The samples were stored at 4 ˚C prior to HPLC analyses.

4.5.5 Polymer Degradation

Chemical hydrolysis of poly(N$_3$-Phe-Ala-Glyc-Lys-propargyl amide). 3 mg of polymer (entry 10) was dissolved in 100 mM Na-phosphate buffer pH 7.0 (1 mL) and incubated at 37 ˚C. After 1, 2, 4, 7 and

23 h a sample was drawn (100 μL) and quenched with ice-cold 1 M Na-acetate buffer pH 3.8 (200 μL). The samples were analyzed by MALDI-TOF.

Enzymatic degradation poly(N_3-Phe-Ala-Lys-propargyl amide). 5 mg of polymer (entry 5) was dissolved in 5 mL 100 mM Tris buffer, pH 7.5 and either 100 μL of α-chymotrypsin in 1 mM HCl (0.5 mg/mL) and 60 μL 2M $CaCl_2$, or 60 μL of trypsin in 1 mM HCl (0.01 mg/mL) was added. The samples were incubated at 37 ˚C in a thermostated water bath. For the ninhydrin assay 250 μL samples were withdrawn at different time points. To inactivate the enzyme, the samples were heated for 5 min at 100 ˚C and immediately stored at –20 ˚C. For mass spectrometric analysis, 100 μL samples were drawn and quenched with 200 μL ice-cold 1M Na-acetate buffer pH 3.8 and immediately stored at –20 ˚C.

Enzymatic degradation poly(N_3-Phe-Ala-Glyc-Lys-propargyl amide). Same procedure as for poly(N_3-Phe-Ala-Lys-propargyl amide), in this case 5 mg of polymer (entry 10) was dissolved in 5 mL 100 mM Na-acetate buffer pH 5.0 and 100 μL of α-chymotrypsin in 1 mM HCl (5 mg/mL) was added . For the degradation with trypsin a 120 μL enzyme solution in 1 mM HCl (0.5 mg/mL) was added to the polymer solution.

4.5.6 Ninhydrin Assay

In a typical experiment, the samples (100 μL) were thawed and diluted with 1M sodium acetate buffer pH 5.5 (900 μL). Next, 1 mL of freshly prepared ninhydrin solution (1.0 g ninhydrin and 150 mg hydrindantin hydrate dissolved in 37.5 mL 2-methoxyethanol and 12.5 mL of 4 M Na-acetate buffer pH 5.5) was added. The mixture was stirred and incubated at 100 ˚C for 15 min. After being cooled to room temperature, the sample was diluted with 5 mL ethanol/water 1:1 v/v and the absorbance was measured at 570 nm. Glycine (in 1 M Na-acetate buffer pH 5.5) was used for calibration.

4.6 References

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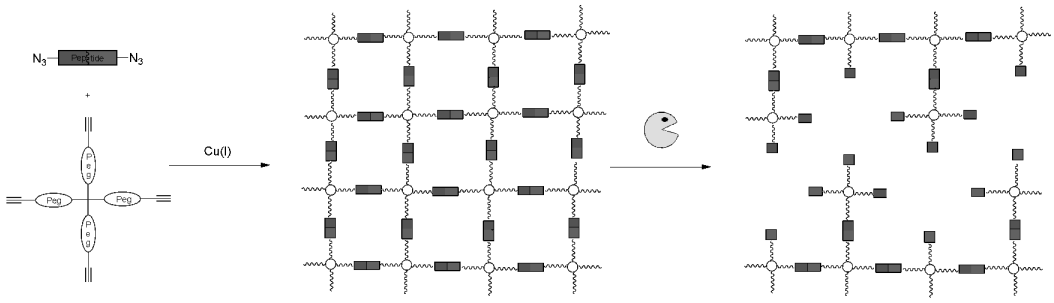
Chapter 5

Synthesis and Characterization of Enzymatically Biodegradable Hydrogels Prepared by Click Chemistry



Maarten van Dijk, Cornelus F. van Nostrum, Wim E. Hennink, Dirk T. S. Rijkers and Rob M. J. Liskamp

5.1 Abstract

This chapter describes the synthesis and characterization of various enzymatically sensitive PEG-based hydrogels by a copper catalyzed 1,3-dipolar cycloaddition reaction. The enzymatically sensitive hydrogels were synthesized from alkyne functionalized star shaped PEG molecules (4-and 8-arm, molecular weights of 10 and 20 kDa) and the diazide-functionalized peptide N^{α}-(azido)-D-alanyl-phenylalanyl-lysyl-(2-azidoethyl)-amide (**1**) in the presence of $CuSO_4$ and sodium ascorbate. The swelling and strength of the hydrogels could be tailored by the initial solid content, the molecular weight of the PEG and also by the architecture of the PEG (4 or 8 arm PEG). The hydrogels were designed to be sensitive for the proteases trypsin and plasmin. For unknown reasons yet, plasmin was not able to degrade the synthesized hydrogels. However, the protease trypsin was able to degrade the hydrogels.

5.2 Introduction

Hydrogels are three-dimensional, hydrophilic polymeric networks capable of absorbing large amounts of water.[1] Over the last past decades, there has been a lively interest in hydrogels, since they can be used for a wide range of applications including drug delivery systems and scaffolds for tissue engineering and repair.[2,3] Hydrogels can be divided into two classes, namely chemically cross-linked and physically cross-linked hydrogels.[4] In chemically cross-linked hydrogels, covalent bonds form the crosslinks which hold together the network, while in physically cross-linked gels the hydrophilic network is hold together by physical interactions like hydrogen bonding, stereo complex formation, hydrophobic and ionic interactions.[5] In general, chemically cross-linked hydrogels have better mechanical properties than physically cross-linked hydrogels. But in physically cross-linked gels the use of toxic cross-linking agent is avoided. Moreover, physically cross-linking is a reversible process, which can be used for loading and release of bioactive substances (e.g. pharmaceutical proteins) and even cells. Chemically cross-linked hydrogels require the introduction of hydrolysis sensitive linkages to allow biodegradability. Most of the biodegradable chemically cross-linked hydrogels rely on hydrolysis of ester bonds present in either the crosslinks or the backbone. Enzymatically degradable hydrogels allow the degradation (and release of the entrapped bioactive compound) to be controlled by cell-secreted and cell-activated enzymes.[6,7] A frequently used method to design enzymatically degradable hydrogels is to introduce small peptide sequences, that are recognized and cleaved by proteases (e.g. trypsin, plasmin and matrix metalloproteinases) in the synthetic hydrogel network.[7-12]
An attractive approach towards the synthesis of degradable hydrogels containing peptides is via the copper(I)-catalyzed cycloaddition (also reviewed in chapter 2).[13-15] This copper(I)-catalyzed 1,3-

dipolar cycloaddition reaction was discovered in 2002 independently by the groups of Meldal[16,17] and Sharpless[18] and is compatible with most functional groups present in proteins and peptides, abolishing the need for protection groups. In addition, the click reaction can be performed in aqueous solution and under mild reaction conditions in terms of pH and temperature, it has high reaction kinetics and gives good product yields, making the click reaction very suitable for the synthesis of hydrogels containing peptidic moieties.

In previous reports we have shown that the copper(I)-catalyzed 1,3-dipolar cycloaddition reaction is an effective tool for the synthesis of (biodegradable) peptide-based polymers.[19,20] Here we want to evaluate the possibilities of the copper(I)-catalyzed 1,3-dipolar cycloaddition reaction for the synthesis of biodegradable hydrogels. In our approach a diazide-functionalized peptide with a trypsin- and plasmin-specific recognition sequence, was used for click reaction with alkyne-functionalized star-shaped PEG molecules to yield hydrogels (Figure 1).

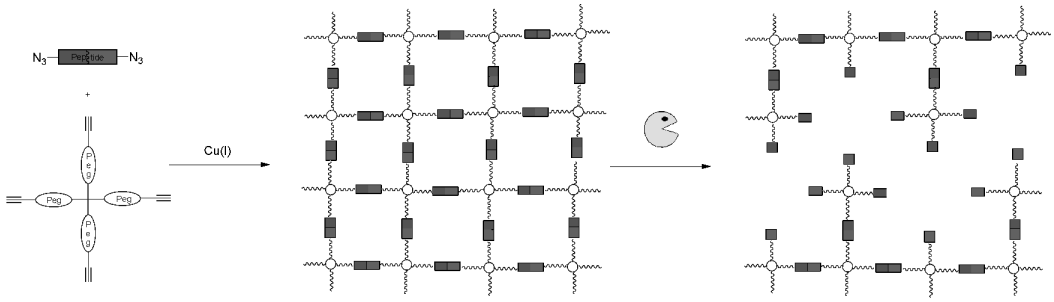


Figure 1: Schematic overview of the synthesis of enzymatically degradable hydrogels synthesized via the copper(I)-catalyzed cycloaddition.

The diazide N^{α}-(azido)-D-alanyl-phenylalanyl-lysyl-(2-azidoethyl)-amide (**1**) (Figure 2) was introduced in the gels as the enzymatically cleavable group. The proteases trypsin and plasmin have a high affinity for this sequence,[21] and both enzymes cleave the amide bond after the lysine residue.[22] In this study three different types of PEG molecules were used, the 4-arm 10 kDa **(6)**, the 4-arm 20 kDa (**7**) and 8-arm 20 kDa **(8)** alkyne-functionalized star-shaped PEG molecules (Figure 3).

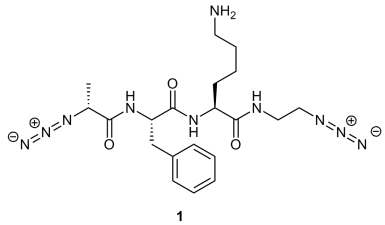


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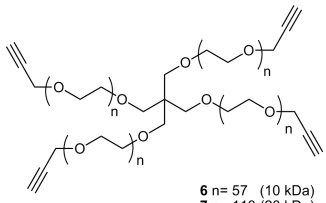
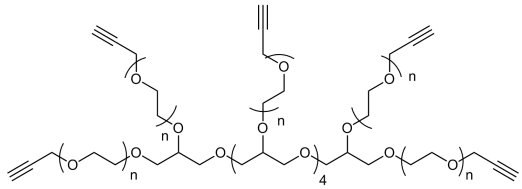
Figure 2: Structure of N^{α}-(azido)-D-alanyl-phenylalanyl-lysyl-(2-azidoethyl)-amide **(1)**.



6 n= 57 (10 kDa)
7 n= 113 (20 kDa)



8 n= 57

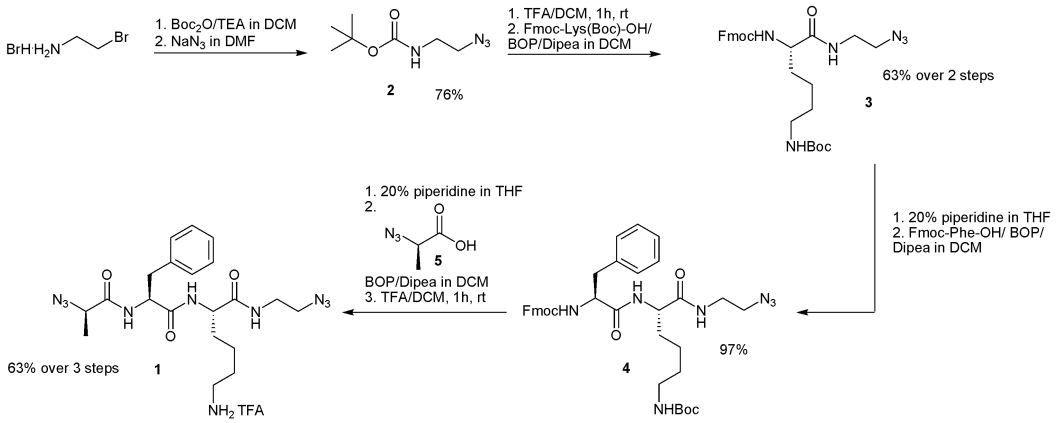
Figure 3: Structure of 4-arm 10 kDa (**6**), 4-arm 20 kDa (**7**) and 8-arm 20 kDa (**8**) alkyne-functionalized star-shaped PEG's.

5.3 Results

5.3.1 Synthesis of di-azido-peptide (1)

The synthesis of N^{α}-(azido)-D-alanyl-phenylalanyl-lysyl-(2-azidoethyl)-amide (**1**) was performed according to Scheme 1 and all synthesis steps proceeded in reasonable to high yields. In short, 2-bromoethylamine hydrobromide was protected with a Boc group. Next, tert-butyl 2-bromoethylcarbamate (**2**) was treated with sodium azide, followed by the removal of the boc protecting group with TFA/DCM to yield 2-azidoethylamine which was subsequently coupled to Fmoc-Lys(Boc)-OH with BOP and Dipea to yield compound (**3**) in 63% yield over two steps. Next, the Fmoc protecting group was removed in the presence of 20% piperidine in THF. Subsequently, Fmoc-Phe-OH was coupled to the lysine residue with BOP and Dipea to yield compound (**4**) in an excellent yield of 97%. Then, after removal of the Fmoc group the dipeptide amine H-Phe-Lys(Boc)-(2-azidoethyl)-amide was obtained, which was coupled to azide **5** in the next step.

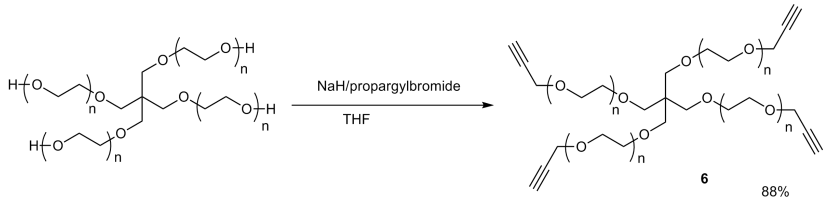
According to a procedure described by Wong et al.[23,24] H-D-Ala-OH was treated with triflic azide in the presence of K_2CO_3 and $CuSO_4 \cdot 5H_2O$ to obtain azido-D-Ala-OH (**5**). Azido-D-Ala-OH (**5**) and the dipeptide H-Phe-Lys(Boc)-(2-azidoethyl)-amide were coupled with BOP and Dipea and after isolatin of the tripeptide the boc group of the lysine side-chain was removed with TFA/DCM to obtain N^{α}-(azido)-D-alanyl-phenylalanyl-lysyl-(2-azidoethyl)-amide (**1**) as a yellow-colored oil in 63% overall yield after purification by column chromatography.



Scheme 1: Synthetic route towards N_3-D-Ala-Phe-Lys-(2-azidoethyl)-amide (**1**).

*5.3.2 Synthesis of alkyne-functionalized star-shaped PEG (**6-8**).*

The alkyne-functionalized star shaped PEGs were synthesized from commercial available hydroxyl-functionalized star shaped PEGs and propargylbromide in the presence of NaH (Scheme 2). Three different alkyne-functionalized star-shaped PEG molecules were synthesized; 4-arm PEG 10 kDa, 4-arm PEG 20 kDa and 8-arm PEG 20 kDa, further referred as PEG_4-10K, PEG_4-20K and PEG_8-20K. The degree of substitution (DS) was determined by ^{1}H NMR spectroscopy, and showed (close to) quantitative substitution based on the intensities of the CH_2 of the PEG and CH_2 of the propargyl signals.



Scheme 2: Synthesis of alkyne-functionalized 4-arm star shaped PEG's.

5.3.3 Preparation and characterization of the hydrogels.

To prepare the hydrogels, N^{α}-(azido)-D-alanyl-phenylalanyl-lysyl-(2-azidoethyl)-amide (**1**), an alkyne-functionalized star-shaped PEG (**6-8**) and sodium ascorbate were dissolved in H_2O and mixed together. For the standard hydrogels a molar ratio alkyne/azide of 1 was used, no efforts were made to exclude oxygen. Subsequently, a solution of copper(II) sulfate in H_2O was added. Upon addition of the copper salt, within minutes a turbid gel was formed. FTIR analysis of the dried hydrogels did not show a signal of the azide functionality (ν 2100 cm^{-1}) (Figure 4C), which was clearly visible in the

FTIR spectra of N^{α}-(azido)-D-alanyl-phenylalanyl-lysyl-(2-azidoethyl)-amide (**1**) (4B), indicating that the click reaction was complete.

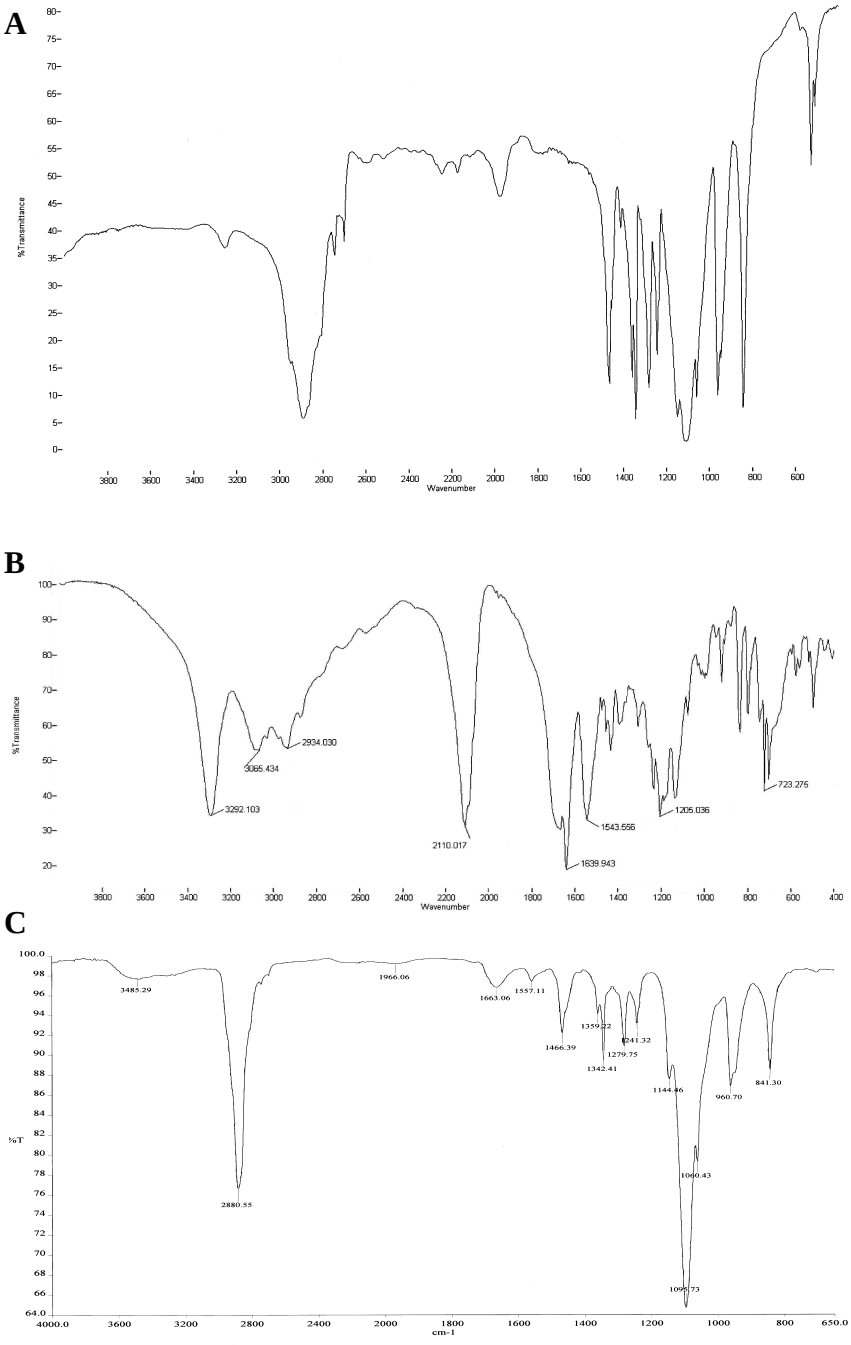


Figure 4: FTIR-spectra of the 8-arm 20 kDa (**8**) alkyne-functionalized star-shaped PEG (A), N^{α}-(azido)-D-alanyl-phenylalanyl-lysyl-(2-azidoethyl)-amide (**1**) (B) and a 10% PEG_4-10K hydrogel (C)

The kinetics of hydrogel formation was followed by rheological measurements. From figure 5 it is clear that upon addition of the copper catalyst to a solution of alkyne functionalized star-shaped PEG, the diazido peptide and sodium ascorbate, a fully elastic hydrogel (G', 22 kPa, tan δ < 0.01) was formed in less than 5 minutes (Figure 5).

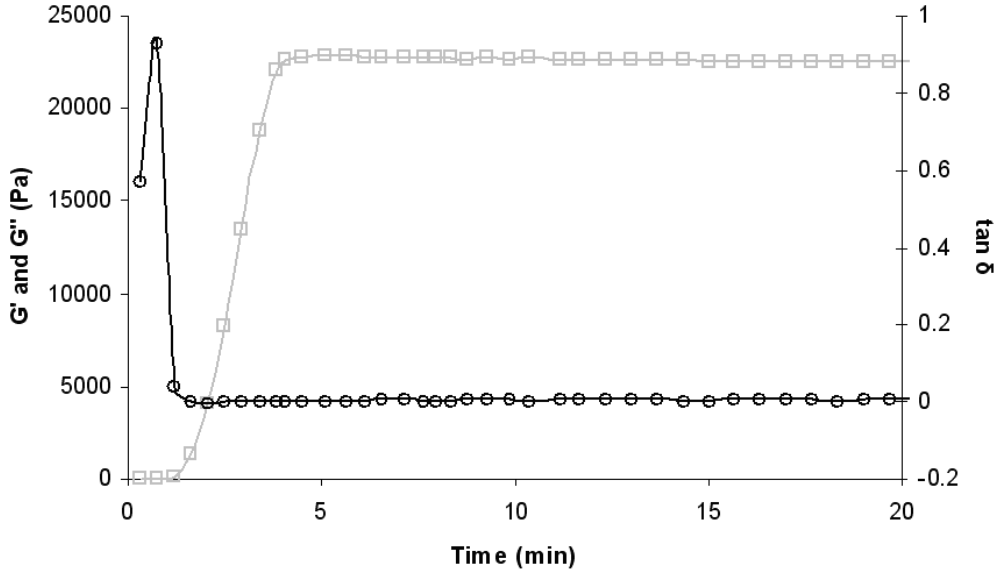


Figure 5: Kinetics of gel formation of 10% PEG_4-10K, in H_2O in which alkyne-functionalized star-shaped PEG_4-10K, N^{α}-(azido)-D-alanyl-phenylalanyl-lysyl-(2-azidoethyl)-amide and ascorbic acid were dissolved G' (□) and tan δ (○) as function of time after addition of $CuSO_4$, azide/alkyne ratio 1/1.

Figure 6 shows that kinetics of gel formation increased with increasing solid content of the hydrogels. A higher solid content gives a higher concentration in reactants, which results in faster reaction rates between the azides and alkynes and as a result the gel point is reached faster. Figure 6 also shows that an increase in solid content resulted in an increased storage modulus (G') of the hydrogel. This is in line with expectations, since a higher solid content results in a higher cross-link density due to higher concentration of reactive groups.[25]

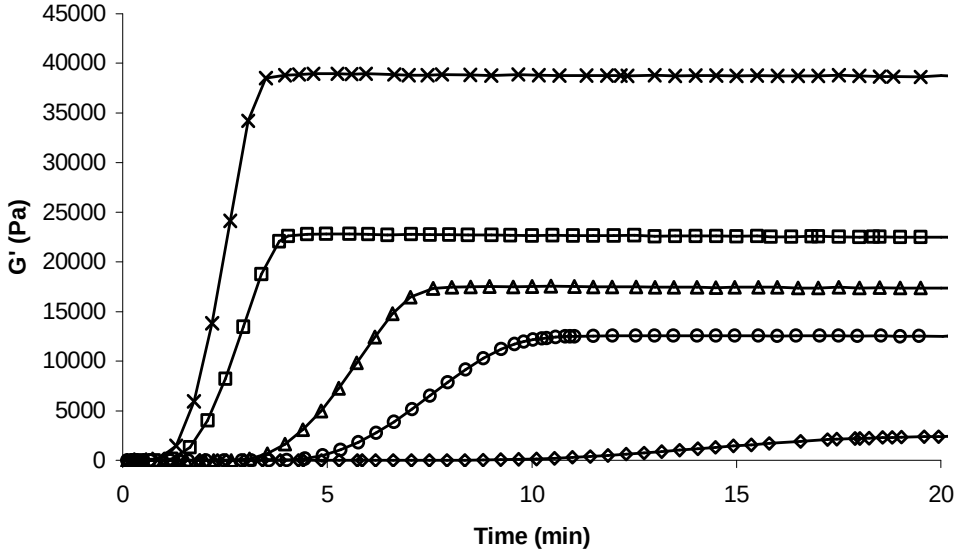


Figure 6: Kinetics of gel formation of 10% PEG_4-10K and N^{α}-(azido)-D-alanyl-phenylalanyl-lysyl-(2-azidoethyl)-amide in H_2O in which ascorbic acid was dissolved at 20 °C as function of time after addition of $CuSO_4$. Storage modulus (G') 2% solid content (◊), 5% solid content(○), 7% solid content (Δ), 10% solid content (□),15% solid content (×). The azide/alkyne ratio was 1.

Besides depending on the solid content, the storage modulus (G') of the hydrogels was also dependent on the type of alkyne-functionalized PEG (Figure 7). The hydrogels based on PEG_4-20K yielded hydrogels with the lowest G' (10% PEG_4-20K, G'= 15.5 ± 0.6 kPa), followed by gels based on PEG_4-10K (10% PEG_4-10K, G'= 24.9 ± 2.3 kPa). This difference can be explained that at equal solid content, the molar concentration of PEG_4-10K and thus the concentration of alkyne groups is twice as high as that of PEG_4-20K. This higher concentration of reactive groups will result in a higher crosslink density and thus higher G'. The hydrogels synthesized with PEG_8-20K had the highest G' (10% PEG_8-20K, G': 39.8 ± 1.0 kPa) (Table 1, Figure 7). The arms of PEG_8-20K and PEG_4-10K have the same length, and consequently both hydrogels have the same molecular weight between cross-links. However, PEG_8-20K has a higher degree of branching and therefore hydrogels derived of this polymer have a higher storage modulus G'.[26]

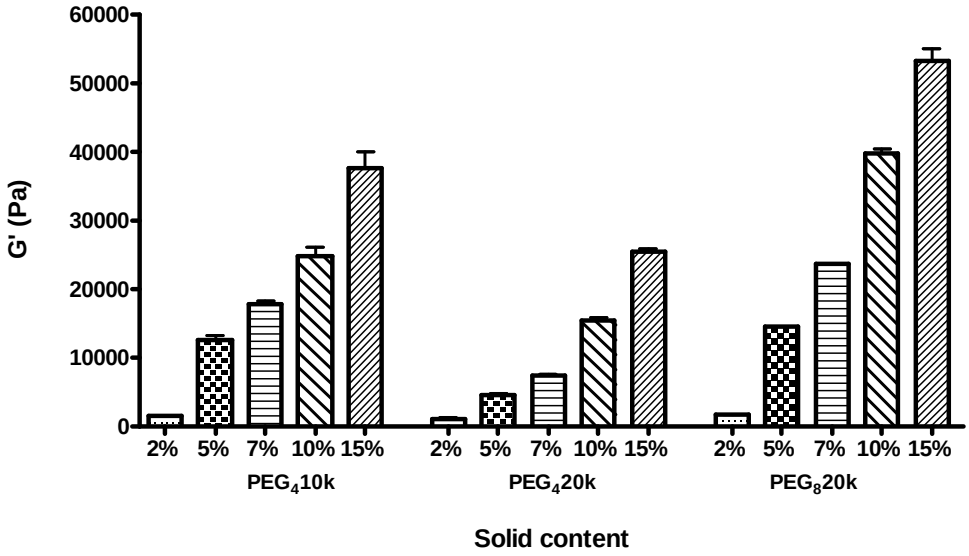


Figure 7: Storage modulus (G') of hydrogels based on PEG_4-10K, PEG_4-20K and PEG_8-20K. The data are shown as average ± SD, n = 3. The gelation reactions were performed with an azide/alkyne ratio of 1 in H_2O at 20 °C.

Table 1: hydrogel characteristics, storage modulus (*G'*), swelling ratio (defined as W_t/W_0), solid content of gel (%)(defined as mass polymer components/ W_t *100), Solid content gel (%) after swelling (defined as mass PEG + peptide/ W_t * 100) and molecular weight between adjacent cross-links (M_c). M_c was calculated from G_0, Theoretical M_c are 5,500 for PEG_4-10K, 10,500 for PEG_4-20K and 5,500 for PEG_8-20K, n.d. not determined The data shown as average ± SD, n = 3

Hydrogel	G' (kPa)	Swelling (W_t/W_0)	Solid content gel (%)	M_c (g/mol)
2% PEG_4-10K	1.4 ± 0.3	n.d.	n.d.	34,900 ± 4,400
5% PEG_4-10K	12.7 ± 0.8	0.96 ± 0.06	5.2	9,600 ± 600
7% PEG_4-10K	17.7 ± 0.8	n.d.	n.d.	9,700 ± 400
10% PEG_4-10K	24.9 ± 2.3	1.50 ± 0.07	6.7	9,900 ± 900
15% PEG_4-10K	37.7 ± 4.2	1.87 ± 0.04	8.0	9,900 ± 1,100
2% PEG_4-20K	1.1 ± 0.1	n.d.	n.d.	44,400± 3,700
5% PEG_4-20K	4.6 ± 0.4	1.79 ± 0.04	2.8	26,600 ± 2,300
7% PEG_4-20K	7.3 ± 0.6	n.d.	n.d.	23,500± 1,800
10% PEG_4-20K	15.5 ± 0.6	2.28 ± 0.10	4.4	15,900 ± 600
15% PEG_4-20K	25.5 ± 0.6	2.92 ± 0.08	5.1	14,600 ± 300
2% PEG_8-20K	1.6 ± 0.2	n.d.	n.d.	30,500 ± 3,400
5% PEG_8-20K	14.4 ± 0.3	0.83 ± 0.05	6.0	8,500 ± 200
7% PEG_8-20K	23.7 ± 0.1	n.d.	n.d.	7,300 ± 100
10% PEG_8-20K	39.8 ± 1.0	1.21 ± 0.03	8.2	6,200 ± 200
15% PEG_8-20K	53.3 ± 3.2	1.64 ± 0.04	9.3	7,000 ± 400

The average molecular weight between adjacent cross-links (M_c) was calculated from the plateau modulus (G_0) using eq 1, derived from the rubber elasticity theory.[27-29]

$$G_0 = \frac{\rho RT}{M_c} \qquad (1)$$

Here, ρ corresponds to the concentration of the polymer solution (g/m^3), R is the gas constant, and T is the temperature (in K). The calculated M_c values were compared with the theoretical M_c values. The theoretical molecular weight between cross-links is equal to twice the molecular weight of each arm of the alkyne-functionalized star-shaped PEG, in addition to the mass of the di-azido-peptide (**1**). Table 1 shows that the calculated M_c values were higher than the theoretical values, most likely due to imperfections in the hydrogels, e.g. the formation of loops, the presence of lose ends.[30] Eq 1 is valid for ideal networks; the networks described in this chapter are not fulfilling the criteria of being ídeal. The deviations in calculation and expected M_c were, more pronounced for gels with a low solid content because more loops and lose ends are created in these systems. Hydrogels based on PEG_8-20K have calculated M_c values that are in close agreement with the theoretical M_c values (PEG_8-20K M_c: 6.2-8.5 kDa, 5.5 kDa expected) (Table 1). These values confirm the conclusion from the FTIR analysis of the hydrogels (Figure 4) that the click reaction was complete.

A series of peptide and PEG (azide/alkyne ratio of 1) were polymerized and monitored via rheology (Figure 8). The highest G' was observed, as expected, around an equimolar ratio of azide and alkyne. When either the number of azide or alkyne groups was in excess, a decrease in G' was found.

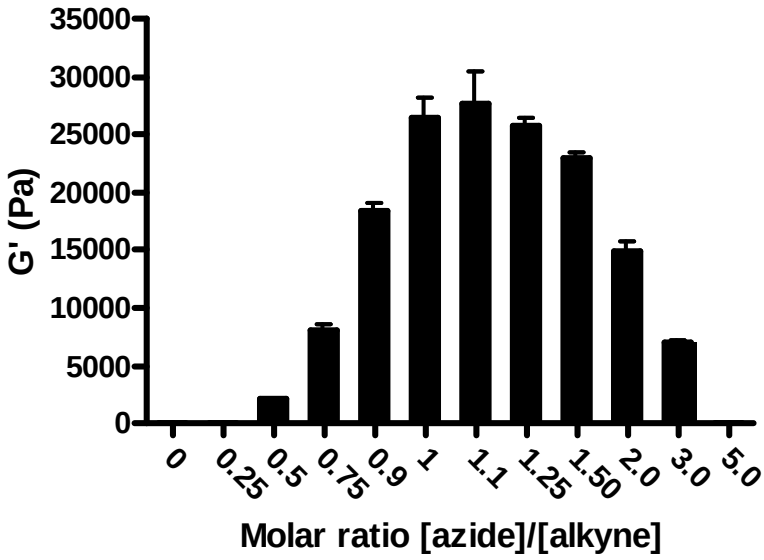


Figure 8: Storage modulus (*G'*) of 10% (w/w) PEG_4-10K hydrogels in H_20 at 20 °C with different azide/alkyne molar ratios. The data are shown as average ± SD, n = 3.

The swelling behavior of the hydrogels was evaluated by incubating the gels in PBS, pH 7.4 at 37 °C. Figure 9A shows that the swelling of hydrogels based on PEG_4-10K reached a plateau within 20 hours. The 5% hydrogels did not increase in weight, but the 10 and 15% gels swelled to respectively 1.5 and 1.8 times their initial weight.

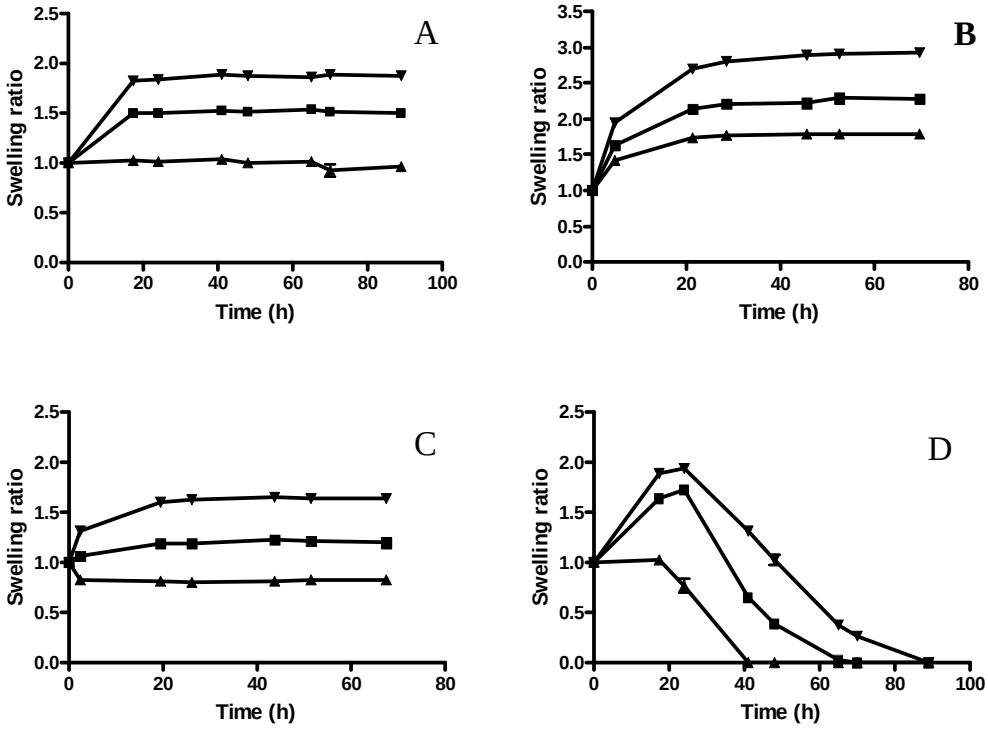


Figure 9: Swelling ratio of hydrogels based on PEG_4-10K (A), PEG_4-20K (B) and PEG_8-20K (C) in PBS buffer pH 7.4 at 37 °C and PEG_4-10K in PBS buffer pH 7.4 at 37 °C containing trypsin (0.8 μM) (D), with different solid content (5% w/w (▲)), (10% w/w (■)) and (15% w/w (▼)). The data are shown as average ± SD, n = 3.

By varying the type of alkyne-functionalized PEG, the swelling properties of the hydrogels can be tailored. Hydrogels based on PEG_4-20K have the highest swelling (figure 9B) which is in line with the rheological analysis (table 1), which shows that hydrogels based on this polymer have the highest M_c and in agreement with previously published papers where it is stated that the degree of equilibrium swelling of a polymeric hydrogel is inversely proportional to hydrogel mechanical strength.[26,31]

For the hydrogels based on PEG_4-10K (Figure 9A) or PEG_8-20K (Figure 9C), only the 10 and 15% gels showed swelling, whereas the 5 % gels were dimensionally stable. Table 1 shows that the M_c of 5% gels is more or less in comparison with the M_c of the initial solid content. Obviously the chains in the 5% gels are already fully stretched and these gels cannot absorb more water. Which is in contrast

to the other 5% gels. The PEG_4-20K gels (Figure 9B) showed some swelling, which can be explained by its higher M_c (Table 1).

5.3.4 Enzymatic degradation of the hydrogels

The synthesized hydrogels have peptides sequences that are known recognition sites for enzymes. For example, the protease trypsin is able to cleave the amide bond after the lysine residue.[22] For the enzymatic degradation reaction, hydrogels were synthesized with three different solid contents (5, 10 and 15%; equimolar azide/alkyne ratio). In the first couple of hours after incubation with the model enzyme trypsin the hydrogels swelled more than the gels incubated in buffer only (Figure 9D). After 20-30 hours (depending on the solid content of the hydrogel) a maximum in swelling was reached. After 30 hours the hydrogels started to loose weight and after 40-80 h the hydrogels were completely degraded (Figure 9D). In line with expectations, the hydrogels with the lowest solid content and as a result with the lowest cross-linking density were degraded most rapidly.

The degradation of the gels was also studied in the presence of the protease plasmin, which is the active form of the zymogen plasminogen. Plasmin is able to degrade fibrin cloths and it also plays a critical role in tumor cell invasiveness and metastasis.[32,33] Plasmin is also present in elevated concentrations in tumor tissues [34] which has been exploited for triggered drug release from plasmin degradable gels.[35]

To study the degradation of the hydrogels by plasmin, they were incubated with this enzyme. It is known that the combination of copper and sodium ascorbate might inhibit plasmin[36] and, therefore, the gels were thoroughly washed with EDTA/PBS buffer prior the incubation with plasmin (2.6 μM). However, even after 200 hours incubation, the weight of the gels remained the same as that of the PBS incubated gels (data not shown). When the peptide N^{α}-(azido)-D-alanyl-phenylalanyl-lysyl-(2-azidoethyl)-amide dissolved in PBS was incubated with plasmin (same concentration as used for the degradation of the hydrogels), the peptide was completely cleaved in 48 h, indicating that the peptide is sensitive for plasmin. Even when a 10-fold amount plasmin was added to the hydrogels no degradation was observed. It might be possible that traces of copper remained in the gel cause inactivation of plasmin.[36] Another possibility is that the peptides sequences in the hydrogel network are inaccessible for plasmin. Plasmin (Mw 75.4 kDa) is much larger than trypsin (Mw 23.8 kDa) and it might be possible that plasmin is unable to access the peptides

5.4 Conclusions

This chapter shows that the copper(I) catalyzed 1,3-dipolar cycloaddition reaction can be used for the synthesis of peptide containing PEG based hydrogels. The hydrogels were rapidly formed under mild conditions, and the storage modulus (G') of the hydrogels could be tailored by varying the solid content, the PEG molecular weight and architecture and by variation of the azide/alkyne ratio. Furthermore, the solid content had influence on the gelation time. The hydrogels had recognition sites in their crosslinks for proteolytic enzymes. For unknown reasons yet, plasmin was not able to degrade the synthesized hydrogels. However, upon incubation of the hydrogels with the protease trypsin, the hydrogels completely degraded within a few days. The degradation time of the hydrogels was dependent on the solid content of the hydrogels.

5.5 Experimental Part

Materials and General Procedures. Chemicals were obtained from commercial sources and used without further purification. Star shaped 4-arm and 8-arm poly(ethylene glycol) (PEG_4-OH and PEG_8-OH) were purchased from JenKem Technology USA (Allen, USA). Reactions were carried out at room temperature unless stated otherwise. Column chromatography was performed with Silica-P Flash silica gel (Silicycle). Thin layer chromatography (TLC) was performed on Merck silica gel 60 F-254 plates. Spots were visualized by UV light, ninhydrin, TDM/Cl_2,[37] $KMnO_4$ or triphenylphosphine/ninhydrin.[38] ^{1}H-NMR spectra were recorded on a Varian Mercury plus 300 MHz and chemical shift values (δ) are given in ppm relative to TMS. ^{13}C-NMR spectra (75 MHz) were recorded using the attached proton test (APT) pulse sequence and chemical shift values are given in ppm relative to $CDCl_3$ (77.0 ppm) or DMSO-d_6 (39.5 ppm). Fourier transform infrared spectra (FTIR) were measured on a Bio-Rad FTS-25 spectrophotometer. Analytical RP-HPLC runs were carried out on a Shimadzu automated HPLC system equipped with a UV/VIS detector operating at λ = 214 and 254 nm and using an Alltech Prosphere C18 column (250 × 4.6 mm, particle size: 5 μm, pore size: 300Å) at a flow rate of 1 mL/min using a linear gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95:5 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 20 or 40 min.

Synthesis of N^{α}-(azido)-D-alanyl-phenylalanyl-lysyl-(2-azidoethyl)-amide (1)

***tert*-Butyl 2-azidoethylcarbamate (2).** 2-Bromoethylamine hydrobromide (16.8 g, 82.7 mmol) and Boc_2O (19.8 g, 91 mmol) were dissolved in DCM (100 mL). The reaction mixture was cooled (0°C) and TEA (12.6 mL, 91 mmol) was added. The reaction mixture was stirred overnight at room temperature. Subsequently, DCM (100 mL) was added to the reaction mixture and the organic layer

was washed three times with 1N $KHSO_4$ (100 mL) and once with brine (100 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated in vacuo. The obtained Boc-protected 2-bromoethylamine was used without further purification in the next step. Yield: 87% (17.70 g) as a white solid. R_f = 0.16 ($CHCl_3$/MeOH/AcOH 95:20:3 v/v/v). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.45 (s, 9H, CH_3), 3.45 (m, 2H, CH_2Br), 3.52 (m, 2H, NHC<u>H</u>$_2$), 5.06 (broad s, 1H, NH), ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 28.3, 32.6, 42.3, 79.7, 155.5. *tert*-Butyl 2-bromoethylcarbamate (17.7 g, 79 mmol) and NaN_3 (10.3 g, 158 mmol) were dissolved in dry DMF (300 mL) and the obtained reaction mixture was stirred at room temperature for 16 hours. Subsequently, the solvent was removed in vacuo, the residue was redissolved in DCM (330 mL) and this solution was washed with 1N $KHSO_4$ (3 × 100 mL) and brine (3 × 100 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated in vacuo. The residue was coevaporated with toluene (3×) and chloroform (3×). *tert*-Butyl 2-azidoethylcarbamate was obtained as a white solid in 87 % yield (12.9 g). R_f = 0.95 ($CHCl_3$/MeOH/AcOH 95:20:3 v/v/v). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.45 (s, 9H, CH_3), 3.31 (m, 2H, CH_2), 3.42 (m, 2H, CH_2), 4.98 (broad s, 1H, NH), ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 28.2, 40.0, 51.1, 79.6, 155.7.

Fmoc-Lys(Boc)-(2-azidoethyl)-amide (3). *tert*-Butyl 2-azidoethylcarbamate (3.9 g, 20 mmol) was dissolved in TFA/DCM 1:1 v/v (80 mL) and the solution was stirred at room temperature for 1 hour. Subsequently, the solvent was evaporated in vacuo. The residue was dissolved in DCM (300 mL) and Fmoc-Lys(Boc)-OH (9.4 g, 20 mmol), BOP (8.8 g, 20 mmol) and Dipea (11 mL, 63 mmol) were added. The reaction mixture was stirred overnight at room temperature. Subsequently, the solvent was removed in vacuo and the residue was dissolved in EtOAc (300 mL). The organic phase was washed with 1 N $KHSO_4$ (3 × 100 mL), H_2O (1 × 100 mL), 5% $NaHCO_3$ (3 × 100 mL), H_2O (1 × 100 mL) and brine (3 × 100 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated in vacuo. The residue was purified by column chromatography with (DCM/MeOH 96:4 v/v) as eluent. Fmoc-Lys(Boc)-(2-azidoethyl)amide was obtained as white solid in 97% yield (10.4 g). R_f = 0.78 ($CHCl_3$/MeOH/AcOH 95:20:3 v/v/v). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.35-1.50 (m, 4H, H^{γ}, H^{δ}), 1.43 (s, 9H, CH_3), 1.65 (m, 1H, H^{β}), 1.86 (m, 1H, H^{β}), 3.10 (m, 2H, H^{ε}), 3.41 (m, 4H, C<u>H</u>$_2$C<u>H</u>$_2$N$_3$), 4.13 (m, 2H, H^{α}, CH Fmoc), 4.40 (m, 2H, CH_2 Fmoc), 4.64 (broad s, 1H, NH urethane), 5.52 (broad s, 1H, NH urethane), 6.57 (broad s, 1H, NH amide), 7.26-7.78 (m, 8H, arom H), ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 22.5, 28.4, 29.5, 31.9, 38.9, 39.7, 47.1, 50.6, 54.8, 67.0, 79.2, 119.9, 125.0, 127.0, 127.7, 141.2, 143.7, 156.2, 172.1, 180.1.

Fmoc-Phe-Lys(Boc)-(2-azidoethyl)-amide (4). Fmoc-Lys(Boc)-(2-azidoethyl)amide (**3**) (10.4 g, 19.3 mmol) was dissolved in 20% piperidine in THF (150 mL). After stirring for 2 h, the solvent was removed in vacuo and the residue was coevaporated with toluene (3×) and chloroform (3×). The

residue was purified by column chromatography with (DCM/MeOH 9:1 v/v) as eluent. H-Lys(Boc)-(2-azidoethyl)amide was obtained as colorless oil in 79% yield (4.8 g). R_f = 0.25 (DCM/MeOH 9:1 v/v). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.37-1.61 (m, 5H, H^β (1H), H^γ (2H), H^δ (2H)), 1.44 (s, 9H, CH_3), 1.87 (m, 1H, H^β), 3.12 (m, 2H, H^ε), 3.34-3.48 (m, 5H, H^α, $C\underline{H}_2C\underline{H}_2N_3$), 4.61 (broad s, 1H, NH urethane), 7.70 (broad s, 1H, NH amide), ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 22.8, 28.4, 29.9, 34.4, 38.6, 40.1, 50.8, 55.0, 79.1, 156.0, 175.3.

H-Lys(Boc)-(2-azidoethyl)amide (4.8 g, 15.2 mmol), BOP (7.1 g, 15.2 mmol) and Fmoc-Phe-OH (5.9 g, 15.2 mmol) were dissolved in DCM (250 mL). Subsequently Dipea (7.9 mL, 45 mmol) was added and the reaction mixture was stirred for 16 hours at room temperature; during the course of the coupling, the reaction mixture turned into a suspension. The suspension was filtered and the residue was dissolved in EtOAc (400 mL) and this solution was subsequently washed with 1 N $KHSO_4$ (4 × 100 mL), H_2O (1 × 100 mL), 5% $NaHCO_3$ (3 × 100 mL), H_2O (1 × 100 mL) and brine (3 × 100 mL). Upon addition of Na_2SO_4 the organic layer transferred into a gel. The gel was separated from the water phase and dried in vacuo. Since the low solubility of the peptide, the crude Fmoc-Phe-Lys(Boc)-(2-azidoethyl)amide was used without further purification in the next step. Fmoc-Phe-Lys(Boc)-(2-azidoethyl)-amide was obtained as a yellow/white solid in 80% yield (8.3 g). R_f = 0.85 ($CHCl_3$/MeOH/AcOH 95:20:3 v/v/v). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.23-1.54 (m, 4H, H^γ, H^δ), 1.41 (s, 9H, CH_3), 1.59 (m, 1H, H^β Lys), 1.83 (m, 1H, H^β Lys), 3.00 (m, 4H, H^β Phe, H^ε), 3.33 (m, 4H, $C\underline{H}_2C\underline{H}_2N_3$), 4.15 (m, 1H,), , 4.28 (m, 1H,), 4.40 (m, 3H,), 4.73 (broad s, 1H, NH urethane), 5.64 (broad s, 1H, NH urethane), 6.80 (broad s, 2H, NH amide), 7.16-7.76 (m, 13H, arom H), ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 22.5, 28.4, 29.3, 31.4, 38.1, 39.0, 47.0, 50.4, 53.1, 56.3, 67.3, 120.0, 124.9, 127.0, 127,7, 128.7, 129.2, 136.1, 141.2, 143.6, 156.2, 171.3, 171.5.

N_3-D-Ala-OH (5). N_3-D-Ala-OH was synthesized according to a procedure described by Lundquist which was based on the method of Wong et al.[23,24] A solution of NaN_3 (18.1 g 279 mmol) in DCM/H_2O (3:2 v/v) (130 mL) was cooled on ice and triflic anhydride (10.0 g, 35 mmol) was added drop-wise in 2 h. The obtained reaction mixture was stirred for 4 h at room temperature. Subsequently, the organic layer was separated and the water layer was extracted twice with DCM (15 mL). The combined organic layers were washed with 5% $NaHCO_3$ (1 × 25 mL) and triflic azide was immediately used in the next step without further purification.

H-D-Ala-OH (1.3 g, 15 mmol), K_2CO_3 (2.9 g, 21 mmol) and $CuSO_4{\cdot}5H_2O$ (29 mg, 0.11 mmol) were dissolved in MeOH/H_2O (2:1 v/v) (90 mL). Triflic azide in DCM was added and the reaction mixture was stirred for 16 at room temperature. Subsequently, the water layer was separated from the organic layer and acidified with 1N $KHSO_4$ till pH 7 was obtained. The water layer was washed with EtOAc (2 × 25 mL) and acidified with 1N $KHSO_4$ till pH 2. Then, the water layer was extracted with EtOAc

(3 × 50 mL). The combined EtOAc layers were concentrated in vacuo and the residue was coevaporated with chloroform (3×). Azido-D-alanine was obtained as yellowish oil in quantitative yield. R_f = 0.59 ($CHCl_3$/MeOH/AcOH 95:20:3 v/v/v). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.55 (d, 3H, H^β), 4.03 (m, 1H, H^α), ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 16.6, 57.0, 177.4.

N^α-(Azido)-D-alanyl-phenylalanyl-lysyl-(2-azidoethyl)-amide (1). Fmoc-Phe-Lys(Boc)-(2-azido ethyl)-amide (**4**) (8.3 g, 12.2 mmol) was dissolved in 20% piperidine in THF (200 mL). After stirring for 2 h, the solvent was removed in vacuo and the residue was coevaporated with toluene (3×) and chloroform (3×). The residue was purified by column chromatography with (DCM/MeOH 9:1 v/v) as eluent. H-Phe-Lys(Boc)-(2-azidoethyl)amide was obtained as yellow oil in 75% yield (4.2 g). R_f = 0.42 (DCM/MeOH 95:5 v/v). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.25-1.68 (m, 6H, H^β Lys (2H), H^γ (2H), H^δ (2H)), 1.44 (s, 9H, CH_3), 2.71-2.79 (dd (J_{ax} 9.1 Hz, J_{ab} 13.8 Hz, 1H, H^β Phe), 3.09 (m, 2H, H^ε), 3.21-3.27 (dd (J_{ax} 4.0 Hz, J_{ab} 13.6 Hz, 1H, H^β Phe), 3.42(m, 4H, $C\underline{H}_2C\underline{H}_2N_3$), 3.65 (m, 1H, H^α Phe), 4.34 (m, 1H, H^α Lys) 4.59 (broad s, 1H, NH urethane), 6.73 (broad s, 1H, NH amide), 7,20-7.36 (m, 5H, arom H), 7.75 (broad d, NH amide), ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 22.6, 28.3, 29.4, 31.7, 38.9, 40.0, 40.7, 50.4, 52.5, 56.1, 78.9, 126.8, 128.6, 129.2, 137.4, 156.0, 171.9, 175.0. N_3-D-Ala-OH (1.2 g, 9 mmol), H-Phe-Lys(Boc)-(2-azidoethyl)amide (4.2 g, 9 mmol) and BOP (4.3 g, 9 mmol) were dissolved in DCM (250 mL). Subsequently Dipea (5 mL, 27 mmol) was added and the reaction mixture was stirred for 16 hours at room temperature. The solvent was removed in vacuo and the residue was dissolved in EtOAc (250 mL). The organic phase was washed with 1 N $KHSO_4$ (4 × 100 mL), H_2O (1 × 100 mL), 5% $NaHCO_3$ (3 × 100 mL), H_2O (1 × 100 mL) and brine (3 × 100 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated in vacuo. The residue was purified by column chromatography with (DCM/MeOH 97:3 v/v) as eluent. N_3-D-Ala-Phe-Lys(Boc)-(2-azidoethyl)amide was obtained as white solid in 84% yield (4.2 g) ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.26-1.64 (m, 7H, H^β Ala, H^β Lys (1H), H^γ, H^δ), 1.43 (s, 9H, CH_3), 1.84 (m, 1H, H^β Lys), 3.08 (m, 4H, H^β Phe, H^ε), 3.41 (m, 4H, $C\underline{H}_2C\underline{H}_2N_3$), 4.00 (m, 1H, H^α Ala), 4.41 (m, 1H, H^α Lys), 4.76 (m, 2H, H^α Phe, NH urethane), 6.80 (broad s, 1H, NH amide), 6.89 (broad s, 1H, NH amide), 7.17-7.77 (m, 6H, arom H (5H), NH amide (1H)), ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 17.0, 22.7, 28.4, 29.5, 32.2, 38.5, 39.0, 40.1, 53.0, 54.1, 58.2, 79.0, 127.0, 128.5, 129.3, 131.1, 136.1, 156.1, 170.6, 170.9, 171.8.

N_3-D-Ala-Phe-Lys(Boc)-(2-azidoethyl)amide (4.2 g, 7.5 mmol) was dissolved in TFA/DCM 1:1 v/v (60 mL) and the solution was stirred for 60 min at room temperature. Subsequently, the solvent was evaporated in vacuo and the crude product was coevaporated with toluene (3×) and chloroform (3×). The crude product was dissolved in water and lyophilized to yield quantitatively N^α-(azido)-D-alanyl-phenylalanyl-lysyl-(2-azidoethyl)-amide (**1**) as a white solid (3.7 g). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.33-1.47 (m, 2H, H^γ), 1.38 (d, 7.2 Hz, 3H, H^β Ala), 1.61-1.70 (m, 2H, H^β Lys (1H), H^δ), 1.82 (m, 1H,

H^{β} Lys), 2.93-3.00 (m, 3H, H^{β} Phe (1H), H^{ε}), 3.11-3.18 (dd, J_{ax} 6.6 Hz J_{ab} 13.8 Hz, 1H, H^{β} Phe), 3.36 (m, 4H, $C\underline{H}_2C\underline{H}_2N_3$), 3.93 (m, 1H, H^{α} Ala), 4.35 (m, 1H, H^{α} Lys), 4.62 (m, 1H, H^{α} Lys), 7.19-7.33 (m, 6H, arom H, NH amide), 7.52 (d ,1H, NH amide), 7.91 (d, 1H, NH amide), 7.98 (broad s, 3H, NH_3), ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 16.7, 21.9, 26.4, 31.0, 37.7, 38.9, 39.3, 50.2, 53.0, 54.7, 58.1, 127.0, 128.5, 129.1, 136.0, 171.3, 171.7, 172.1. ESI-MS calcd for $C_{20}H_{30}N_{10}O_3$: 458.25 found m/z $[M+H^+]$ = 459.20.

Alkyne-functionalized star-shaped PEG (6). In a typical procedure**,** 4-arm star-shaped PEG-OH (Mw 10.000) (10.2 g, 1 mmol) was dissolved in dry THF (150 mL). Subsequently, NaH (420 mg as a 60% suspension in mineral oil, 10.5 mmol) was added and the reaction mixture was stirred at room temperature. After 15 min, propargyl bromide (600 μL, 80% in toluene, 5.4 mmol) was added and the reaction mixture was stirred for 16 hours at room temperature. Subsequently, the solvent was removed in vacuo and the residue was dissolved in H_2O (150 mL) and dialyzed against H_2O (MWCO 3.500). The product was lyophilized to yield alkyne-functionalized star shaped PEG as a white solid in 88% yield (8.8 g). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 2.44 (t, 1H, C≡CH), 3.39-3.75 (m, H, CH_2), 3.83 (m, 2H, $C\underline{H}_2C{\equiv}CH$), ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 35.0, 64.0, 68.9, 76.4.

Preparation of hydrogels. In a typical gelation experiment (10 % gel, 10 kDa 4-arm PEG), 220 μL of a solution that consists of alkyne-functionalized star shaped PEG (1350 mg), N^{α}-(azido)-D-alanyl-phenylalanyl-lysyl-(2-azidoethyl)-amide (141 mg) and sodium ascorbate (135 mg) dissolved in 4.5 mL H_2O was transferred in an open syringe and diluted with 270 μL H_2O. Subsequently, 63 μL of $CuSO_4$ (100 mg $CuSO_4{\cdot}5H_2O$ in 10 mL H_2O) was added. No efforts were made to exclude oxygen. The solution was mixed and within minutes a turbid gel was formed.

Rheological Characterization. Rheological characterization of the hydrogels was done with an AR G-2 rheometer (TA Instruments, Etten-Leur, The Netherlands) equipped with 2° steel cone geometry of 40 mm diameter and solvent trap. Upon addition of the $CuSO_4$ solution to the solution of PEG/peptide/Na-ascorbate in water, 600 μL of the solution was placed between the plates of the rheometer. Rheological gel characteristics were monitored by oscillatory time sweep experiments. During time sweep experiments the *G'* (shear storage modulus) and *G''* (loss modulus) were measured in the oscillation mode with a controlled strain of 0.1% at a frequency of 1 Hz at 20 °C for a period of 30 min.

Swelling behavior of hydrogels. In a typical swelling experiment the gels were weighted (W_0) and incubated in 10 mL 100 mM PBS, pH 7.4, containing 0.3 mg/mL NaN_3 (to prevent bacteria growth)

and incubated at 37˚C. To determine the swelling ratio (defined as W_t/W_0) the gels were removed from buffer at certain time intervals and weighed (W_t). The buffer was replaced by a fresh buffer every 24 h.

Degradation experiments. The gels were incubated with 0.1 M EDTA solution for 24 h, followed by several washing with PBS. Subsequently, the gels were incubated in 5 mL 100 mM PBS, pH 7.4, containing 0.3 mg/mL NaN_3 and after 1 day of incubation the gels were weighted (W_0) and incubated in either 5 mL 100 mM PBS, pH 7.4, containing 0.3 mg/mL NaN_3 or the same buffer with plasmin (2.6 μM) or trypsin (0.43 μM). At different time points, the gels were removed from buffer and weighed (W_t). The buffer was replaced by a fresh buffer containing enzyme every 24 h.

To study the peptide degradation, 3 mg of peptide was dissolved in 1 mL PBS pH 7.4 and trypsin (50 μL, 0.01 mg/mL) dissolved in 1 mM HCl or plasmin (20 μL, 1 mg/mL) dissolved in water.was added. The samples were incubated at 37˚C in a thermostated water bath. At different time points, 100 μL of sample was drawn and quenched with 200 μL ice-cold 1M Na-acetate buffer pH 3.8. The samples were stored at 4 ˚C prior to HPLC analyses.

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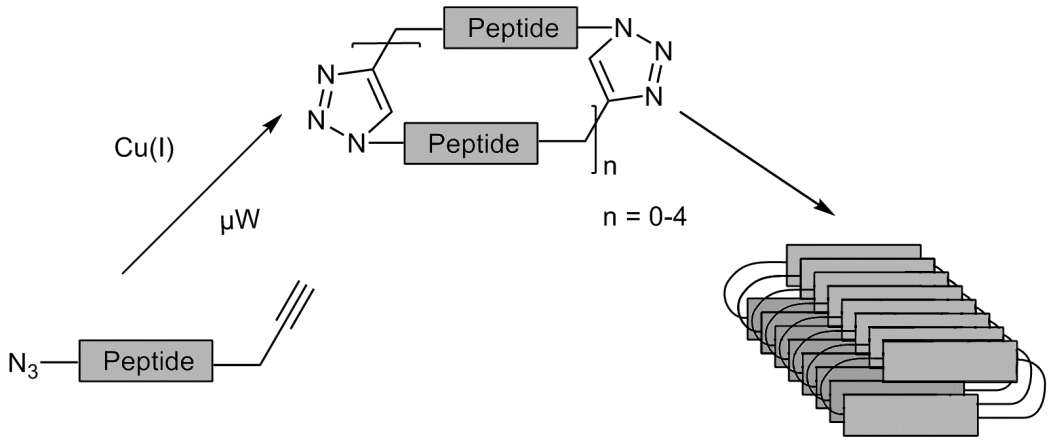
Chapter 6

Microwave-assisted Click Polymerization for the Synthesis of Aβ(16-22) Cyclic Polymers



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6.1 Introduction

The development of peptide-based oligomers and polymers with unique self-assembly properties has been ongoing for many years now. Application of such oligomers and polymers are often found in drug delivery systems, medical devices, tissue engineering and catalysis.[1-4] Interest in the field of biomaterials is strongly emerging since peptide-based polymers have been made biocompatible towards cells and human tissue. Furthermore, there is an increasing interest in *de novo* design of small molecules, which are able to self-assemble into large supramolecular polymeric constructs since these small building blocks are easier to synthesize.

Several examples of small molecules like cyclic peptides, which are able to self-assemble into supramolecular structures such as peptide nanotubes, have been reported in the literature. Ghadiri was one of the first to demonstrate that cyclic peptides, prepared from alternating L- and D- amino acids, were able to form peptide nanotubes via hydrogen bonding.[5-8] Since then, several groups have reported on the self-assembly of cyclic peptides.[9] Reches and Gazit have used the hydrophobic Phe-Phe dipeptide core recognition motif of Alzheimer's Aβ peptide to prepare peptide nanotubes[10,11] and Lynn and co-workers found that a peptide based on Aβ(16-22) self-assembles into peptide nanotubes at low pH.[12-14] In general terms, amyloidogenic peptides have been chosen since they have a molecular self-recognition function, which is an intrinsic tendency to self-assemble and therefore might direct assembly of peptides into desired supramolecular constructs.

From chapter 3 and 4 it is known that azido-alkyne peptides can be converted into polymers containing up to 300 amino acid residues using the Cu(I)-catalyzed Huisgen 1,3 dipolar cyclo-addition reaction or "click" reaction.[15,16] It was also shown in chapter 3 that a variation of the concentration of azido-alkyne monomer product formation could be directed to either cyclic or linear polymers. Since the development of the copper catalysed "click reaction" it has been used for, among others, the preparation of cyclic peptides. The triazole ring which is formed during the cycloaddition reaction, may not only promote cyclization, but is also a good mimic of a peptide bond.[17,18]

It was hypothesized that the click reaction could facilitate the synthesis of the designed cyclic triangular shaped peptide construct. Therefore, amyloidogenic Aβ(16-22) was converted into its azide-alkyne congener: N_3-Lys-Leu-Val-Phe-Ala-Glu-propargyl amide (peptide **1**, Figure 1) and polymerized using the click reaction.

The Aβ(16-22) peptide is a highly amyloidogenic peptide sequence which rapidly forms anti-parallel β-sheets in aqueous solution. Exploration of β-sheet formation might result in novel material properties of the Aβ(16-22) oligomers. Small cyclic peptide (trimers and tetramers) were of specific interest to be synthesized and studied because of their possible self-assembly properties *(vide supra)*

(Figure 2). In addition, hydrophobic and electrostatic interactions could promote self-assembly at an appropriate pH. Under physiological conditions, lysine and glutamic acid are charged and could be beneficial for electrostatic cross recognition of oligomers in the process of self-assembly.

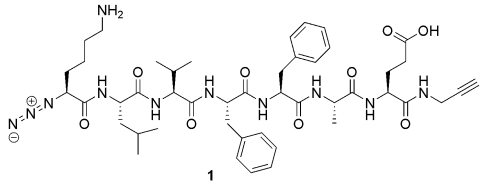

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Figure 1: The Aβ(16-22) peptide converted into its azide-alkyne congener.

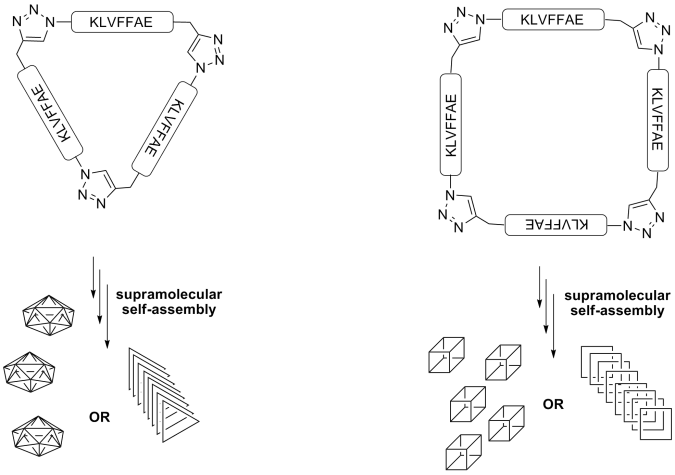
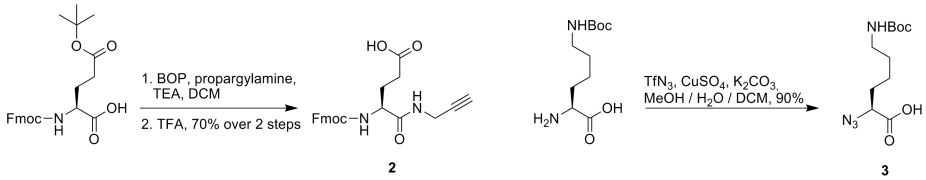


Figure 2: Cross-recognition of amyloid peptide sequences in designed peptide triangles and squares.

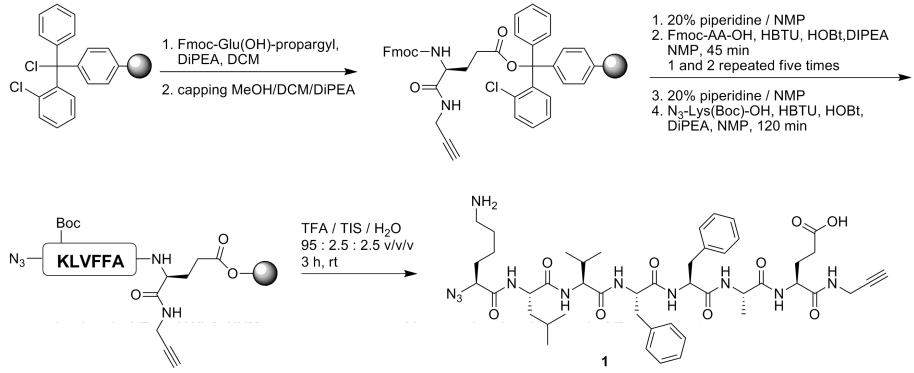
6.2 Results and Discussion

To facilitate preparation of the peptide, the alkyne- and azido amino acid building blocks **2** and **3** respectively, were synthesized in solution. Alkyne monomer **2** was synthesized from commercially available Fmoc-Glu(O*tBu*)-OH. First, propargylamine was coupled to the carboxylic acid moiety, followed by cleavage of the *tert*-butyl group under acidic conditions. Subsequently, N_3-Lys(Boc)-OH **3** was prepared according to the method of Lundquist and Pelletier (Scheme 1).[19,20]



Scheme 1: Synthesis of alkyne- and azide building blocks.

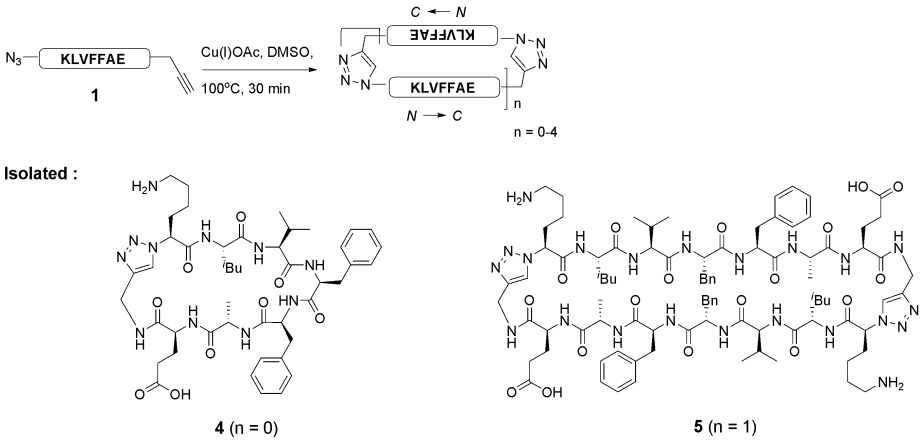
Next, peptide **1**, which served as a "monomer" for the click polymerisation reaction, was synthesized in a straightforward manner using Fmoc/*t*Bu chemistry (Scheme 2). The peptide was synthesized on a polystyrene resin functionalized with a 2-chloro trityl chloride linker.[21] The resin was loaded with compound **2**, capped with methanol and base to deactivate any residual trityl chloride. The loading of the resin was determined to be 0.46 mmol/g. Then, synthesis of peptide **1** was continued using Fmoc/*t*Bu chemistry, ending with the coupling of compound **3**. Peptide **1** was cleaved from the resin using TFA, TIS and H_2O as cleavage and deprotection mixture (Scheme 2). The crude product was precipitated in cold MTBE/hexane and subsequently lyophilised to give peptide **1** in a satisfactory yield and purity. It was characterized by electro spray mass spectroscopy and its purity was analysed by HPLC. Analysis with infrared spectroscopy showed an absorption at 2107 cm^{-1} which confirmed the presence of the azide functionality.



Scheme 2: Synthesis of azido-alkyne peptide **1**.

Next, monomer **1** was polymerized using the Cu(I) catalyzed 1,3 dipolar cycloaddition reaction. It is known that amyloidogenic peptide **1** has a strong tendency to aggregate, therefore compound **1** was dissolved in degassed dimethylsulfoxide and CuOAc was added under a nitrogen atmosphere. The click reaction was performed using microwave irradiation for 30 min at 100°C (Scheme 3)[15,16], after which the crude reaction mixture transformed to a gel.

The crude reaction mixture was analysed by MALDI-TOF and revealed the presence of at least four different compounds (Figure 3). Based on the molecular weight of peptide **1** (916.50 Da) it turned out that the peaks in the mass spectrum corresponds to a dimer (1831.54 Da), trimer (2749.34 Da), tetramer (3663.65 Da) and pentamer (4581.45 Da). Purification by preparative HPLC resulted in the isolation of cyclic monomer **4** (14%) and cyclic dimer **5** (53%). Since the characteristic azide- and alkyne adsorptions were absent in the infrared spectrum it was concluded that the isolated compounds were cyclic. Although the formation of tri-, tetra- and pentamers was proved by MALDI-TOF, isolation by preparative HPLC was not successful.



Scheme 3: Synthesis of cyclic oligomers of Aβ(16-22).

The higher abundance of cyclic dimer **5** might be explained by a mechanism recently proposed by Finn and coworkers.[22,23] The mechanism assumes that the favoured dimerization involves the formation of a dialkyne complex ligated to Cu(I), which is followed by an energetically more favoured *exo*-like cyclization.[22-24] Furthermore, the 1,4 substituted triazole functionality has been described as a good β-turn inducing moiety and therefore promotes the anti-parallel organization of the formed dimer, which ultimately leads to the formation of the cyclic dimer.[25,26]

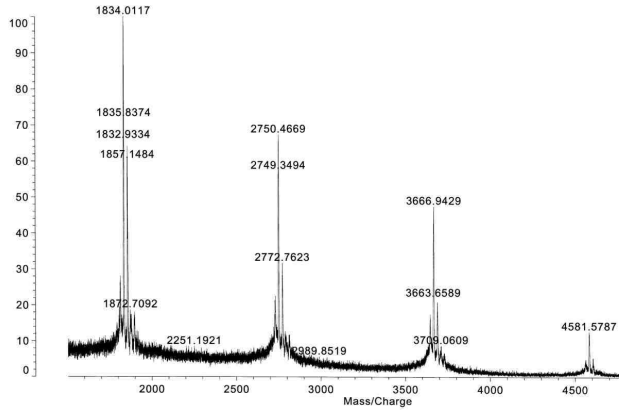


Figure 3: MADLI-TOF spectrum of the crude reaction mixture after the click reaction of peptide **1**.

Next, the physical and self-assembling properties of the obtained cyclic peptides **4** and **5** were studied. For this purpose, a concentrated stock solution of each individual peptide was prepared in DMSO which was diluted ten times in H_2O (to a final concentration of 6 mg/mL, pH 7) This solution was aged for three weeks to initiate self-assembly of the cyclic peptides. As a comparison, peptide **1** was treated in the same manner. After this period of incubation, the morphology of supramolecular assemblies was visualized using transmission electron microscopy (TEM). The presence of any secondary structure was investigated using infrared spectroscopy and circular dichroism.

For peptide **1**, no gel formation was observed. Analysis by TEM however, showed the presence of lamellar sheet-like assemblies (Figure 4A). Infrared spectroscopy revealed an absorption at 1628 cm^{-1} which indicated that these lamellar sheets consisted of anti-parallel β-sheets.[27,28] This result is in agreement with the literature since it was shown that *N*-terminal acylation resulted in an anti-parallel assembly of Aβ(16-22).[29]

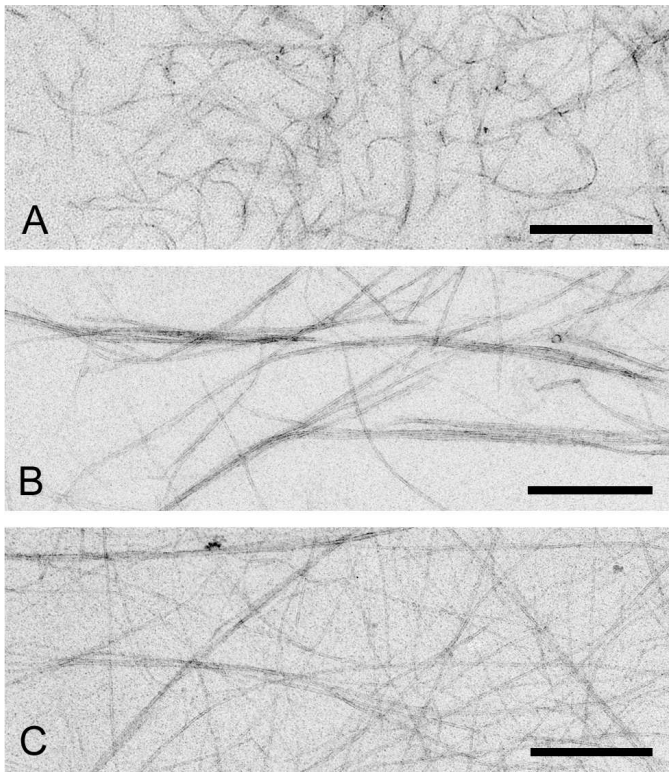


Figure 4: TEM pictures of peptide **1** (A), peptide **4** (B) and peptide **5** (C). Scale bars represent 500 nm.

For peptide **4**, also no gel formation was observed. However, in this case TEM analysis revealed the presence of many broad fibre-like assemblies (a width up to 80 nm was observed, Figure 4B). These supramolecular constructs displayed different morphologies compared to peptide **1** (Figure 4A). Analysis by Infrared spectroscopy indicated an absorption at 1672 cm^{-1}. The shift in wave number from 1628 to 1672 cm^{-1} might be explained by assuming that self-assembly is based on hydrophobic interactions and π-π- interactions (Phe) or electrostatic interactions (Glu/Lys) rather than hydrogen bond formation via the peptide backbone. Moreover, CD-analysis showed a positive absorption at 193 nm and a negative absorption at 205 nm, which is also an indication that self-assembly of cyclic peptide **4** is not caused by β-sheet formation (Figure 5).

Peptide **5** gelled the solution after three weeks of aging. Clearly, peptide **5** behaved differently compared to **4** since differences in morphology and its CD-spectrum were observed. Analysis by electron microscopy revealed the presence of fibres or fibril-like entities, which were thinner and longer compared to those formed by peptide **4** (Figure 4C). The CD-spectrum of **5** (Figure 5) showed a positive absorption at 195 nm and a negative absorption at 230 nm, typical features of a β-sheet conformation. The presence of a β-sheet was confirmed by FTIR analysis since characteristic

absorptions at 1636 cm^{-1} and 1686 cm^{-1} were observed which were an indication for the presence of an intra/inter strand hydrogen bond pattern.

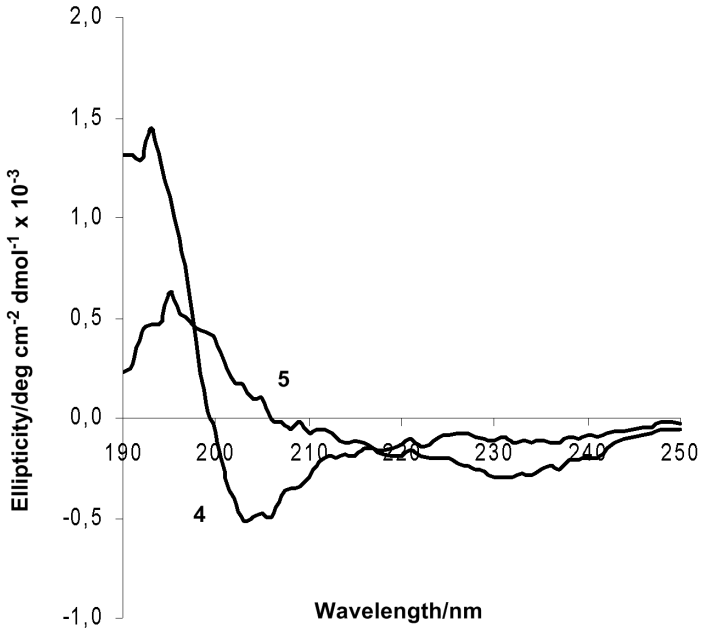


Figure 5: Circular dichroism spectra of peptide **4** and peptide **5** in HFIP/H_2O 1:9 v/v (concentration 0.3 mg/mL).

Based on these experimental data the following models are proposed that explain the molecular interactions of peptide 4 respectively 5. Peptide 4, the cyclic monomer in which a triazole is incorporated as part of the backbone, is represented as its lowest energy conformation in which a single intramolecular hydrogen bond is found between the NH of the former propargylamide and the carboxyl oxygen of Phe20, as shown in Figure 6A. The absence of the amide I absorption at 1628 cm^{-1} strongly indicates that the self-assembly of peptide 4 into lamellar sheets is primarily based on electrostatic interactions between the lysine en glutamic acid side chains and π-π- interactions via the phenylalanine side chains, as is schematically represented in Figure 6B.

Since a triazole ring is a known β-turn inducer and in combination with the IR-data, the anti-parallel β-sheet as depicted in Figure 7 is proposed as a plausible model of peptide 5. In this model, intrastrand hydrogen bonds as well as interstrand hydrogen bonds are in close agreement with the observed amide I absorption at 1636 cm^{-1}. Moreover, intermolecular interactions of two cyclic dimers can be the result of hydrogen bonding (intrastrand interactions) and electrostatic interactions via the lysine and glutamic acid side chains. Unfortunately, a lowest energy conformation of this cyclic peptide could not be obtained due to its high flexibility.

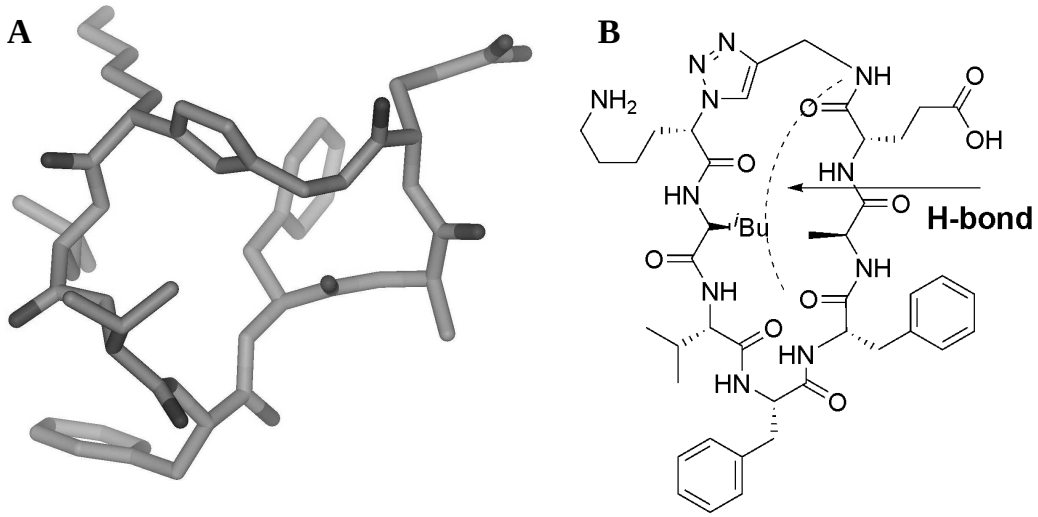


Figure 6: A lowest energy conformation of peptide **4** (Macromodel 7.0). B: Model for the self-assembly of peptide **4**.

The cyclic trimer and tetramer as depicted in Figure 1 could not be isolated in their pure form by preparative HPLC although an indication of their formation was given by MALDI-TOF. The triangular and square shapes of these peptide constructs are rather speculative especially since the cyclic dimer 5 is too flexible to calculate a lowest energy conformation. Nevertheless, it has been shown that the structure of the peptide linear (**1**), cyclic monomer (**4**) and cyclic dimer (**5**) is an important factor which determines the molecular basis of the self-assembly of these peptides leading to different morphologies varying from lamellar sheets to amyloid-like fibrils.

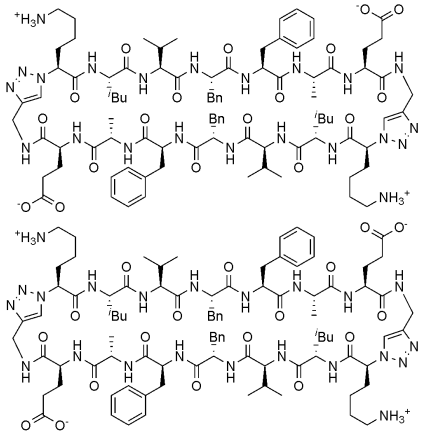


Figure 7: Model of self-assembly of peptide **5.**

6.3 Conclusions

In conclusion, an efficient and facile synthesis of an extended azido/alkyne (Aβ16-22)-derived peptide was described. In the presence of Cu(I)OAc, the azido/alkyne peptide could be oligomerized via a microwave-assisted 1,3-dipolar cycloaddition reaction into cyclic constructs containing up to five repeating units (35 amino acid residues) as judged by MALDI-TOF analysis. Two cyclic peptides (cyclic monomer 4 and cyclic dimer 5) were isolated by HPLC. Higher weight oligomers could not be isolated probably due to aggregation. The self-assembly and aggregation behaviour of peptides 4 and 5 in water was studied by electron microscopy, infrared spectroscopy and circular dichroism. Striking differences in the role of hydrogen bonds with respect to the self-assembly of both peptides were found. These differences were most pronounced regarding the morphology of the supramolecular assemblies ranging from lamellar sheets to amyloid-like fibrils.

6.4 Experimental methods

General procedures: Chemicals were obtained from commercial sources and used without further purification. Peptide grade solvents used for solid phase peptide synthesis were stored on 4Å molecular sieves. Microwave reactions were performed in a Biotage initiator apparatus with pressure and temperature control. ^{1}H-NMR spectra were recorded at 300 MHz or at 500 MHz and chemical shift values (δ) are given in ppm relative to TMS. ^{13}C-NMR spectra (75 MHz) were recorded using the attached proton test (APT) pulse sequence and chemical shift values are given in ppm relative to DMSO (39.5 ppm). Peptides were characterized using Electro Spray Mass Spectrometry (ESI-MS) on a Finnigan LCQ Deca XP Max apparatus operating in a positive ionization mode. MALDI-TOF analyses were performed on a Kratos Axima CFR apparatus, with ACTH(18-39) as an external reference (monoisotopic [M + H]+: 2,465.1989 Da) and sinapinic acid as matrix. Electron microscopy was performed at 60 kV.

Synthesis of Fmoc-Glu(OH)-propargyl amide (2): Fmoc-Glu(tOBu)-OH (4.3 g, 10 mmol), propargylamine hydrochloride (915 mg, 10 mmol) and BOP (4.4 g, 10 mmol) were dissolved in CH_2Cl_2 (100 mL) and DIPEA (3.6 mL, 21 mmol) was added. The obtained reaction mixture was stirred for 16 h at room temperature. Then, the solvent was removed under reduced pressure and redissolved in EtOAc (150 mL). The organic solution was washed with 1N $KHSO_4$ (2 × 75 mL), saturated $NaHCO_3$ (2 × 75 mL) and brine (50 mL). The organic layer was dried (Na_2SO_4) and subsequently concentrated in vacuo. The obtained reaction product was used without further

purification in the next reaction step. Crude Fmoc-Glu(tBu)-propargyl amide was dissolved in a mixture of CH_2Cl_2/TFA (100 mL 1:1 v/v) and stirred for 2h at room temperature. Subsequently, the reaction mixture was concentrated and coevaporated with CH_2Cl_2 to remove any residual TFA. Then, the product was purified by column chromatography (eluent: MeOH/CH_2Cl_2 5:95 v/v). Amide **2** was obtained as a white solid in 70% yield (2.8 g). Mp. 151-154 °C; R_f: 0.2 (MeOH/CH_2Cl_2 5:95 v/v); ^{1}H-NMR (300 MHz, $CDCl_3$/CD_3OD 9:1 v/v) δ: 2.10-1.86 (double m, 2H, βCH_2 Glu), 2.30 (m, 1H, propargyl), 2.38 (m, 2H, γCH_2 Glu), 4.00 (s, 2H, CH_2 propargyl), 4.23 (m, 2H, CH Fmoc, 1H α-CH Glu), 4.40 (m, 2H, CH_2 Fmoc), 7.78-7.30 (m, 9H, NH Fmoc, CH Fmoc (1H + 8H), 7.97 (s, 1H, NH propargyl) ^{13}C-NMR (75 MHz, $CDCl_3$/CD_3OD 9:1 v/v) δ: 27.5, 28.6, 29.7, 46.8, 53.6, 66.7, 71.2, 78.7, 119.6, 124.7, 126.8, 127.5, 141.0, 143.4, 156.5, 171.6, 175.2; ESI-MS calcd for $C_{23}H_{22}N_2O_5$: 407.1607 found m/z [M+H$^+$] = 407.1626.

Synthesis of N_3-Lys(Boc)-OH (3). N_3-Lys(Boc)-OH was synthesized starting with commercially available H-Lys(Boc)-OH according to protocols described by Lundquist IV.[19,20] Azide **3** was obtained as a slightly yellowish oil in 90% yield (2.94 g). Analysis data were in agreement with literature.

Solid phase peptide synthesis of N_3-Lys-Leu-Val-Phe-Phe-Ala-Glu-propargyl amide (1): A polystyrene resin functionalized with a 2-chloro Trityl linker (1 g, initial loading: 1 mmol/g) was loaded with Fmoc-Glu-(OH)-propargyl amide (1.2 g, 3 mmol) in DCM (10 mL) in the presence of DiPEA (1.6 mL, 9 mmol) for 16 h. Subsequently, unreacted trityl moieties were capped with methanol (CH_2Cl_2/MeOH/DiPEA 17:2:1 v/v/v, 2 × 10 mL, 20 min). The amount of Fmoc-Glu(OH)-propargyl amide coupled to the resin was determined by an Fmoc-determination according to Meienhofer[30] and was found to be 0.46 mmol/g. The peptide sequence was synthesized using Fmoc/^{t}Bu SPPS protocols on a 0.25 mmol scale. The N-terminal N_3-Lys(Boc)-OH was coupled with HBTU/HOBt (2 equiv) and DIPEA (4 equiv) during 2h. The coupling reactions were monitored with the Kaiser test.[31] The peptide was deprotected and cleaved from the resin with TFA/TIS/H_2O (95:2.5:2.5 v/v/v) and precipitated in MTBE/hexane (1:1 v/v) at –20°C. The crude peptide was lyophilized from *tert*-BuOH/H_2O (1:1 v/v). Peptide **1** was obtained in 74% yield (310 mg).; ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 0.71 (d *J* = 4.4 Hz, 6H, δ/δ' CH_3 Leu), 0.84 (dd, 6H, γ/γ' CH_3 Val), 1.21 (d *J* = 7.2 Hz, 3H, CH_3 Ala), 1.53 (m, 1H, β-CH Val), 1.73-1.30 (m, 6H, β, γ,δ-CH_2 Lys), 1.86-1.73 (m, 2H, β-CH_2 Glu), 1.86-1.73 (m, 3H, β-CH_2 Leu, γ-CH Leu), 2.22 (m, 2H, γ-CH_2 Glu), 2.75 (m, 2H, ε-CH_2 Lys), 3.05-2.68 (m, 4H, β-CH_2 Phe (2 × 2H)), 3.12 (m, 1H, CH propargyl), 3.77 (t, *J* = 6.9 Hz, 1H, α-CH Lys), 3.85 (m, 2H, CH_2 propargyl), 4.08 (t, *J* = 7.8 Hz, 1H, α-CH Leu), 4.38-4.23 (m, 3H, α-CH Ala, Val, Glu), 4.53 (m, 2H, α-CH Phe (2 × 1H)), 7.24-7.18 (m, 10H, arom H Phe (2 × 5H)), 7.83 (d *J* = 8.8 Hz, 1H, NH Leu), 8.00-7.93 (m, 2H, NH Phe + NH Glu), 8.11 (d *J* = 8.0 Hz, 2H, NH

Phe), 8.18 (d *J* = 7.2 Hz, 2H, NH Ala), 8.37-8.30 (m, 2H, NH propargyl amide, NH Val); ESI-MS calcd for $C_{46}H_{65}N_{11}O_9$: 916.50 found m/z [$M+H^+$] = 916.97.

Polymerization of N_3-Lys-Leu-Val-Phe-Phe-Ala-Glu-propargyl amide (1). We have shown before that lower concentrations of monomer led to more cyclic products.[15] Therefore, monomer **1** (40 mg, 0.043 mmol) was dissolved in N_2-purged dimethylsulfoxide (2 mL), and CuOAc (3 mg, 0.025 mmol) was added. The reaction mixture was placed in the microwave reactor and irradiated at 100 °C for 30 min. The clear solution was transformed into a turbid gel. The gel was dissolved in additional DMSO (3 mL) and transferred in small tubes. The solvent was evaporated in vacuo (speedvac.). The obtained solid was dissolved in H_2O/CH_3CN (1:1 v/v) and lyophilized. The crude mixture of oligomers was obtained in quantitative yield (40 mg). The different oligomers were separated by preparative HPLC. Peptide **4** was obtained as a white powder in 5.6 mg yield (14%) and peptide **5** was obtained as a white powder in 13 mg yield (33%).

Peptide purification. Preparative HPLC runs were carried out on an Applied Biosystems 400 Semi Automated HPLC System equipped with an Applied Biosystems 757 UV/VIS Absorbance Detector (λ = 214 nm). The crude lyophilized peptides were dissolved in a minimum amount of TFA and loaded onto an Alltech Prosphere C4 column (250 × 22 mm, particle size: 10 mm, pore size: 300Å). The peptides were eluted with a flow rate of 10 mL/min using a linear gradient of buffer B (75% in 120 min) from 100% buffer A (buffer A: 0.1% TFA in CH_3CN/H_2O 5:95 v/v, buffer B: 0.1% TFA in CH_3CN/H_2O 95:5 v/v).

Transmission Electron Microscopy. The peptide gel/solution (6 mg/mL) aged for three weeks and 10 μL was placed on a carbon coated copper grid. After 15 min, any excess of peptide was removed by washing the copper grid on a drop of demi-water (this was repeated five times). Finally, the samples were negatively stained by methylcellulose/uranyl acetate and dried on air.

Circular Dichroism. CD spectra were measured at 1.0 nm intervals in the range of 190-250 nm as the average of 10 scans using a spectral band width of 1.0 nm in 2 mm cuvettes thermostated at 20°C with the optical chamber continually flushed with dry N_2 gas. The spectra were measured in HFIP at a concentration of 0.3 mg/mL. A peptide sample was dissolved in HFIP/H_2O 1:9 v/v and measured instantly.

Fourier transform infrared spectroscopy. A peptide gel/solution aged for three weeks (100 μL) was lyophilized and subsequently resuspended in D_2O (150 μL) and lyophilized. This treatment was

repeated twice. A peptide sample was mixed with KBr and pressed into a pellet. The optical chamber was flushed with dry nitrogen for 1 min before data collection started. The interferograms from 128 scans with a resolution of 2 cm^{-1} were averaged and corrected for H_2O and KBr.

Computational modelling.[14,32,33] A lowest energy conformation of peptides **1** and **4** were performed on a SiliconGraphics O_2 workstation with MacroModel 7.0 using the organic builder and the peptide builder in the grow mode. MMFF94 was used as a force field. After construction, the structures were minimized on an Origin200 Server using Molecular Mechanics calculations with the following settings: MMFF94 (GB/SA water solvent model, planar N's),[14] MCMM (Monte Carlo Multiple Minimum, conformational search), PCRG, CCrit 0.01 kJ/molÅ, iterations > max. RCG: Conjugate gradient minimization using the Polak-Ribiere first derivative method with restarts every 3N iterations. Should not find saddle points. Best general minimization method for energy minimization. BatchMin code for carrying out this method is highly vectorized for efficient operation on vector hardware.[32] Iterations/stop, sets the maximum number of iterations Batchmin will use to energy minimize a structure.

Finally, a conformational search was carried out starting with the minimized structure using a Monte Carlo run which generated 1000 structures (all appropriate single bonds will become variable, all double bonds, amide bonds and ester bonds will become constrained, potential chiral centers will be set and flexible rings will be opened). The goal of the conformational searching was to locate the low-energy conformations of the structure of interest. The settings of a standard conformational search: Monte Carlo Multiple Minimum (MCMM); Number of steps: 1000; Solvent: H_2O. After each MCMM step, the structure was minimized again (with the same settings as above). Lowest energy conformation for peptide **1**: -56.046 kJ/mol; peptide **4**: +6.372 kJ/mol. The structure of peptide **5** could not be minimized by MacroModel 7.0 due to the presence of too many flexible bonds.

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Chapter 7

Thermoresponsive Methacryloylamide Polymers Based on L-Serine and L-Threonine Alkyl Esters

Polymers with tailorable cloud points and degradation kinetics

Maarten van Dijk, Tobias Postma, Cornelus F. van Nostrum, Wim E. Hennink, Dirk T. S. Rijkers, and Rob M. J. Liskamp

7.1 Abstract

This chapter describes the design and synthesis of amino acid-based thermoresponsive polymers with tailorable cloud points and degradation kinetics. Six monomers were synthesized, based on the methyl, ethyl and isopropyl esters of N^{α}-methacryloyl- L-serine and L-threonine. The methacryloylated monomers were polymerized by AIBN-initiated radical polymerization and yielded polymers with M_n's between 6.6 and 23.8 kDa. The obtained homo- and co-polymers showed thermosensitive behavior in aqueous solution with a broad range of cloud points, e.g. between 1.5 and 100 °C. The CP decreased with increasing hydrophobicity of the polymers. The degradation kinetics of the monomers was investigated to obtain insight into the stability of the polymers in aqueous solutions. According to HPLC analysis the half-life time of the methyl esters was 5 days, 12 days for the ethyl ester derivatives and around 40 days for the isopropyl esters. The degradation rate of poly(N^{α}-methacryloyl-Ser-OMe) and poly(N^{α}-methacryloyl-Thr-OMe) was determined by NMR and proved to be a factor 4 lower compared to the corresponding monomers. Upon hydrolysis of the ester bond a carboxylic acid group is formed which increases the hydrophilicity of the polymers, and as a result of this, an increase in polymer cloud point was observed. In order to obtain thermoresponsive micelles, the monomer N^{α}-methacryloyl-Thr-OEt was polymerized using $(PEG)_2$-ABCPA as macroinitiator to yield a block co-polymer, poly(N^{α}-methacryloyl-Thr-OEt)-b-PEG 5000, which forms in aqueous buffer particles with a size of 300 nm above its cloud point of 24°C. Upon incubation in a physiological buffer system (pH 7.4, 37°C) the ester groups in poly(N^{α}-methacryloyl-Thr-OEt)-b-PEG 5000 were hydrolyzed resulting in destabilization of the micelles.

7.2 Introduction

Thermoresponsive polymers with a lower critical solution temperature (LCST) are soluble in aqueous environment below a certain temperature, referred to as cloud point (CP). Once the temperature of the polymer solution reaches the CP, phase separation occurs and the polymer precipitates. Below the CP, the polymer chains are hydrated and consequently in a dissolved state. Above the CP, hydrogen bonds between the polymer and water are disrupted leading to dehydration of the polymer chains, which results that hydrophobic moieties interact and the polymer precipitates.[1]

Thermoresponsive polymers with a CP around human body temperature are under investigation for biomedical and pharmaceutical applications.[2,3] For clinical applications, thermoresponsive polymers must be biocompatible and biodegradable. Three classes of thermoresponsive polymers can be distinguished: non-biodegradable, side-chain degradable and main-chain degradable, which will be discussed in more detail in the next sections.

7.2.1 Non-biodegradable thermoresponsive polymers

The biocompatibility of non-biodegradable thermoresponsive polymers is questionable and therefore the clinical use of these polymers is limited, especially when the molecular weight of the polymer is above the renal threshold value (typically 30 kDa).[4,5] Examples of these polymers are: poly(*N*-isopropyl acrylamide) (PNIPAAm, CP: 32 °C),[6] poly(*N*-acryloyl-*N*'-propylpiperazine) (PAcrNPP, CP: 37 °C),[7] poly(*N*-vinyl-isobutyramide) (PNVIBA, CP: 39 °C) and poly(vinyl methyl ether) (PVME, CP: 35 °C) (Figure 1).[8]

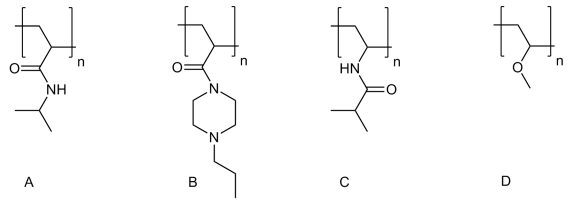


Figure 1: Structure of PNIPAAm (A), PAcrNPP (B), PNVIBA (C), PVME (D).

7.2.2 Thermoresponsive polymers with degradable side groups

Thermoresponsive polymers that contain biodegradable side-chains have been designed in order to overcome the previous mentioned limitations of their non-biodegradable counterparts. Neradović et al.[9,10] proposed a novel concept, in which a thermoresponsive polymer *N*-isopropyl acrylamide (NIPAAm) was co-polymerised with 2-hydroxyethyl methacrylate-monolactate (HEMA-monolactate). By increasing the HEMA-monolactate content the CP the LCST of the polymers decreases. Upon hydrolysis of the lactate side groups the LCST of the polymer increases. Depending on the polymer composition it is possible to design polymers that are initially insoluble at body temperature, but will dissolve in time due to hydrolysis of the lactate side groups. This was also shown for poly(N-(2-hydroxypropyl) methacrylamide mono/di lactate) **(**poly**(**HPMAm-mono/di lactate)), of which the CP exceeded 37 °C in 5 days (for the dilactate) under physiological conditions.[11-13]

Amino acid ester-based acrylamide polymers have been shown to exhibit thermoresponsive behaviour in aqueous environment. By incorporation of amino acid residues onto the polymer backbone, highly ordered structures, such as α-helices and β-sheets, can be formed by non-covalent interactions as for instance hydrogen bonding.[14] The thermoresponsive behaviour depends on the hydrophilic and hydrophobic balance of a polymer. The acrylamide backbone is hydrophobic and therefore hydrophilic amino acids, such as D,L-alanine[15], L-alanine[16] and L-proline[17-19] (Figure 2) are used. Ester groups were introduced by esterification of the amino acid moiety, which changes the

hydrophilic/hydrophobic balance and lead to partially biodegradable polymers. Esters hydrolyse under physiological conditions and therefore amino acid ester acrylamides are expected to show a change in CP in time.

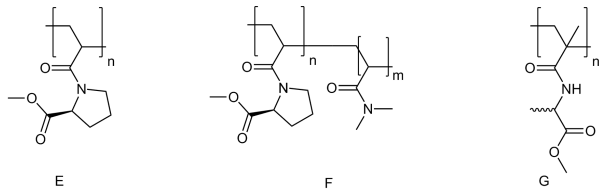


Figure 2: Structure of: poly(N^{α}-acryloyl-L-proline methyl ester) (E), poly(N^{α}-acryloyl-L-proline-OMe-co-DMA) (F), poly(N^{α}-methacryloyl-DL-alanine methyl ester) (G).

7.2.3 Thermoresponsive polymers with a biodegradable main chain

Recently, biodegradable thermoresponsive polymers have been developed with biodegradable moieties in the main chain of the polymer.[20] The formed degradation products are either eliminated by excretion via the kidneys or metabolized. Enzymatic digestion and chemical hydrolysis are the most common degradation pathways for the degradation of polymers; the former essentially applies to biopolymers whereas the latter is the degradation route for synthetic polymers.[21] Amino acid-based polymers are used as fully biodegradable thermoresponsive polymers. Some examples of amino acid-based biodegradable thermoresponsive polymers are: poly(Glyc-Asn(isopropyl)) (CP: 29 °C),[22] poly(***N*^α^**-substituted γ-glutamines co-polymer) (CP: 21-50 °C) ,[23,24] poly(γ-glutamic acid-co-γ-glutamic acid propyl ester) (CP: 25-83 °C).[25] (Figure 3)

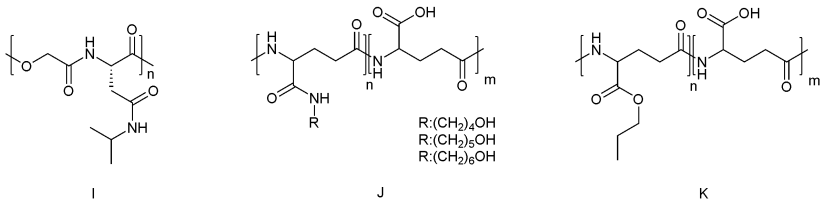


Figure 3: Structure of: poly[Glyc-Asn(isopropyl)] (I), poly(N^{α}-substituted γ-glutamine) (J), poly(γ-glutamic acid-co-γ- glutamic acid propyl ester) (K).

7.2.4 Thermoresponsive polymers in drug delivery

Thermoresponsive polymers are widely investigated for their possible application in the biomedical and pharmaceutical field.[26] In conventional drug application, the drug is introduced into the body as a free compound, which leads to non-selective biodistribution, degradation of the drug and unpredictable detrimental side effects. Thermoresponsive polymers for example, can be used to encapsulate drugs and to change their pharmacokinetic properties by reducing the degradation rate of

the drug. By introducing biodegradable moieties in the thermoresponsive polymers, the drug can be released in a specified time and with a controlled release pattern.[27] In addition, thermoresponsive polymers can be used as component for nanosized polymeric micelles. The small size of these nanosized polymeric micelles allows them to accumulate in tumors (enhanced permeation and retention effect).[28,29] Thermoresponsive polymers for drug delivery applications must be non-toxic, biocompatible and biodegradable. Furthermore, thermoresponsive polymers should preferably have a CP below body temperature because this allows utilization of the phase transition to load drugs. For example, biodegradable thermosensitive polymers with degradable side groups were used as a hydrophobic part of an amphiphilic polymer. When this amphiphilic polymer dissolved in water and incubated at 37 °C, micelles are formed and the drug is entrapped inside the hydrophobic core of the micelle. Upon hydrolysis of the biodegradable moieties, the micelles slowly desintegrate and the drug is released (Figure 4). This was shown for poly(HPMAm-dilactate)-co-PEG, in which the CP changed from below to above body temperature and led to complete dissolution of the micelles in 7 days.[30]

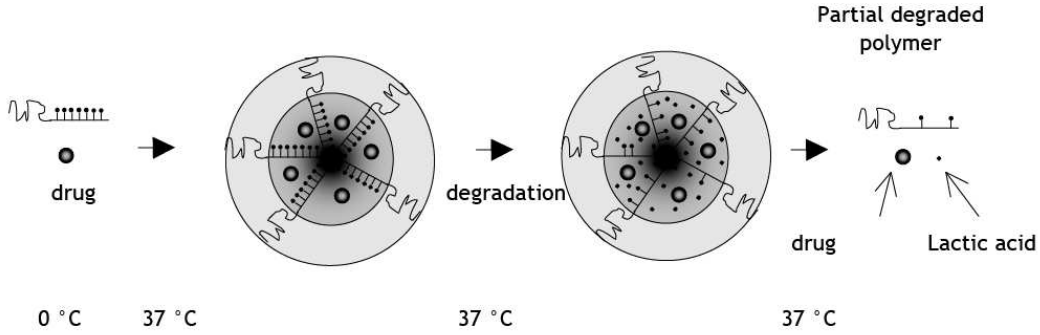
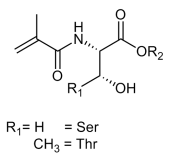


Figure 4: Example of biodegradable thermosensitive polymer as drug delivery systems.[31]

The aim of this chapter was the design and synthesis of novel thermoresponsive polymers with tailorable cloud point and degradation kinetics for biomedical and pharmaceutical applications. The cloud point and degradation kinetics of the polymers should be easily altered by small modifications of the polymer. Furthermore, the polymers should contain biodegradable moieties, which can function as a release mechanism for entrapped drugs. For this purpose methacryloylamide polymers based on L-serine and L-threonine esters were chosen. These polymers were expected to have tailorable thermoresponsive behavior, which was likely to be achieved by altering the hydrophilic/hydrophobic balance by the choice of monomers (i.e. L-serine or L-threonine) and the choice of esters. Tailorable degradation kinetics was aimed for the choice of the ester moiety, namely methyl-, ethyl- and isopropyl esters, respectively (Figure 5).



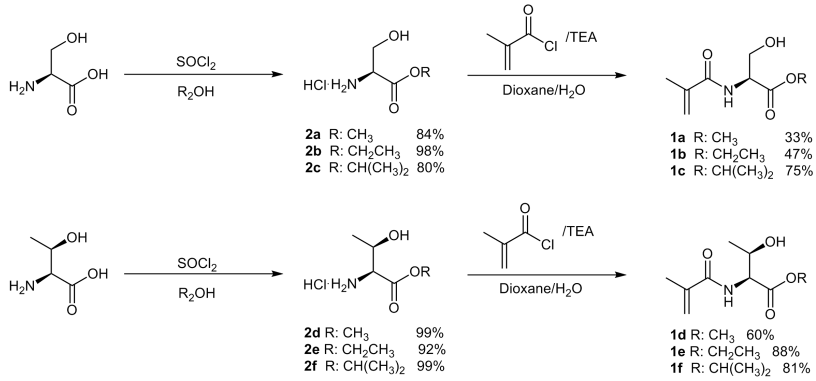
	R_1	R_2
1a	H	Me
1b	H	Et
1c	H	*i*Pr
1d	CH_3	Me
1e	CH_3	Et
1f	CH_3	*i*Pr

Figure 5: The six monomers that have been synthesized in this study: N^{α}-methacryloyl-Ser-OMe (MA-Ser-OMe) (**1a**), N^{α}-methacryloyl-Ser-OEt (MA-Ser-OEt) (**1b**), N^{α}-methacryloyl-Ser-O*i*Pr (MA-Ser-O*i*Pr) (**1c**), N^{α}-methacryloyl-Thr-OMe (MA-Thr-OMe) (**1d**), N^{α}-methacryloyl-Thr-OEt (MA-Thr-OEt) (**1e**), N^{α}-methacryloyl-Thr-O*i*Pr (MA-Thr-O*i*Pr) (**1f**).

7.3 Results and Discussion

7.3.1 Monomer synthesis

The monomers **(1a-f) (**Scheme 1**)** were synthesized in a two step approach as shown in Scheme 1. In the first step, the aimed esters were synthesized by reacting L-serine or L-threonine with the corresponding alcohol in the presence of thionyl chloride (5 equiv.) (overall yields of 80 to 99%). In the second step, the amino acid esters of L-serine or L-threonine were treated with methacryloyl chloride under Schotten-Baumann conditions with Et_3N as base (pH 8.5 to 9). The crude methacryloyl amino acid esters were purified by column chromatography to give monomers (**1a-f**) in 33-88% yield (Scheme 1). The monomers were analyzed by NMR and HPLC and had a purity over 98%.



Scheme 1: Synthesis of monomers **(1a-f)**.

7.3.2 Polymerization Reactions and Cloud Point Determinations

The synthesized monomers were polymerized via a free radical polymerization with 2,2'-azobisisobutyronitrile (AIBN) as radical initiator. The reactions were preformed over a 48 h period at 70 °C in deoxygenated and dry DMF with a monomer/initiator (M/I) ratio of 100/1 (mol/mol). After the polymerization reaction, the solvent was evaporated and the crude product was redissolved in chloroform, followed by precipitation in hexane. The polymers were obtained in 82 to 96% yield as

white solids. The physical properties of the synthesized polymers are shown in Table 1. The M_n values ranged form 9.8 kDa to 15.7 kDa. Furthermore, the molecular weight distribution was rather small for polymers synthesized by classical free radical polymerization (polydispersity indices between 1.6 and 2.0). It is known that polymers below 30 kDa can be excreted from the body through renal clearance and therefore accumulation of methacrylamide polymers can be avoided by molecular weight control.[32]

Interestingly, the homo-polymers showed a broad range of cloud points (in the range from 1.5 to 65 °C; Table 1). The homo-polymers with the methyl ester had the highest CP, follow by the ethyl ester polymers. The isopropyl esters, which are the most hydrophobic groups, had the lowest CP. Furthermore, in line with expectations, the more hydrophobic threonine-based polymers had a lower CP than the serine-based polymers. As expected, the cloud point decreased with increasing hydrophobicity of the polymer (Table 1).

To further fine-tune the CP, several co-polymers of various monomers were synthesized. These co-polymers were synthesized with a 1:1 co-monomer ratio and had CPs between those of the homo-polymers. For most copolymers the measured CP was close to the predicted CP based on the molar ratios of the monomers. Summarizing, the results of table 1 demonstrate that thermoresponsive methacryloylamide polymers based on L-serine and L-threonine esters are not only tailorable by the type of amino acid ester but also by co-polymerizing the various monomers.

Table 1: Characteristics of the synthesized polymers.[†]

Entry	Synthesized polymers*	M_n[a] (kDa)	M_w[a] (kDa)	Yield (%)	PDI[C]	CP[d] (°C)	Hyst[e](°C)	Predicted CP*** (°C)
	Homo-polymer							
3	poly(MA-Ser-OMe)	15.7	29.7	82	1.9	>100	-	-
4	poly(MA-Thr-OMe)	12.3	22.4	90	1.8	64.5	2.0	-
5	poly(MA-Ser-OEt)	14.4	25.0	96	1.7	49.5	10.0	-
6	poly(MA-Thr-OEt)	12.6	20.2	84	1.7	24.0	2.0	-
7	poly(MA-Thr-OEt)[f]	6.6	12.1	81	1.8	24.5	1.0	-
8	poly(MA-Thr-OEt)[g]	23.8	46.7	78	2.0	19.5	1.0	-
9	poly(MA-Ser-O*i*Pr)	13.8	26.9	90	1.9	6.5	3.0	-
11	poly(MA-Thr-O*i*Pr)	9.8	16.7	88	1.7	1.5	-	-
	Co-polymer (1:1 co-monomer ratio)**							
12	poly(MA-Ser-OMe-co-MA-Thr-OMe)	16.2	26.2	88	1.6	83.0	5.0	>82
13	poly(MA-Ser-OMe-co-MA-Ser-OEt)	13.0	22.6	50	1.7	81.0	4.0	>75
14	poly(MA-Ser-OMe-co-MA-Thr-OEt)	14.6	26.3	83	1.8	46.0	2.0	>62
15	poly(MA-Ser-OMe-co-MA-Ser-O*i*Pr)	23.4	45.3	65	1.9	32.0	2.0	>53
16	poly(MA-Ser-OMe-co-MA-Thr-O*i*Pr)	13.2	23.6	80	1.8	26.0	1.0	>50
17	poly(MA-Ser-O*i*Pr-co-MA-Thr-OMe)	13.3	25.0	91	1.9	25.0	0.5	35
18	poly(MA-Ser-O*i*Pr-co-MA-Ser-OEt)	13.7	25.3	78	1.8	24.8	1.0	28
19	poly(MA-Thr-O*i*Pr-co-MA-Ser-OEt)	14.0	22.7	89	1.6	17.0	1.0	33
20	poly(MA-Thr-O*i*Pr-co-MA-Thr-OMe)	18.2	33.4	95	1.8	14.0	1.5	25
21	poly(MA-Ser-O*i*Pr-co-MA-Thr-OEt)	16.3	30.0	75	1.7	14.0	1.0	14
22	poly(MA-Thr-O*i*Pr-co-MA-Thr-OEt)	17.8	29.8	88	1.7	10.5	1.5	13
23	poly(MA-Thr-O*i*Pr-co-MA-Ser-O*i*Pr)	15.2	24.8	32	1.6	6.0	3.6	4

[†]The polymers are listed on CP. [a]M_n and M_w were determined by GPC with 10 mM LiCl in DMF as an eluent and PEG standards for calibration; [c]PDI: polydispersity index (M_w/M_n), [d]CP cloud point, [e]Hyst: thermohysteresis.. [f](M/I 50/1). [g](M/I 200/1).

* Polymerizations were conducted at an M/I ratio of 100/1, unless stated otherwise

** The final copolymer composition could not be determined by NMR, due to broadening and overlap of the signals.

*** Predicted $CP_{co\text{-}polymer} = CP_{homo\text{-}polymer}$ 1 * molar fraction monomer 1 + $CP_{homo\text{-}polymer}$ 2 * molar fraction monomer 2.

For poly(*N*-isopropyl acrylamide) (PNIPAAM)[33] and poly(2-n-propyl-2-oxazoline) (PnPropOx)[34] the CP depends on the molecular weight of the polymers. It was shown that a higher molecular weight resulted in a lower CP. Therefore, the effect of the polymer molecular weight on the cloud point of poly(MA-Thr-OEt) synthesized with an M/I ratio of 200/1, 100/1 and 50/1 was investigated (Table 1). Poly(MA-Thr-OEt) synthesized at an M/I ratio of 50 (M_n = 6.6 kDa, M_w = 12.1 kDa), had only a slightly higher CP (24.5 °C) than the polymer synthesized at an M/I ratio of 100 (M_n = 12.6 kDa, M_w = 20.2 kDa, CP= 24 °C). The CP of the polymer synthesized at an M/I ratio of 200 (M_n = 23.8 kDa, M_w = 46.7 kDa) was significant lower (19.5 °C) than the other two polymers.

Upon cooling of the polymer dispersion, it was shown that the CP is reversible. However, the cloud point was generally found to be several degrees lower for all polymers tested (Table 1, Figure 6). The decrease of cloud point temperature, referred to as thermohysteresis, is explained by the presence of an alpha carbon on the polymer backbone, which increases the polymer rigidity.[13]

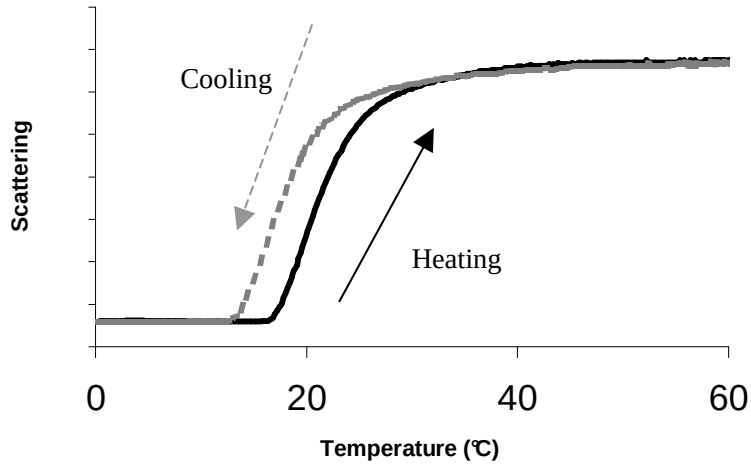


Figure 6: Thermohysteresis for poly(MA-Thr-OEt) at 1 mg/ml in H_2O (λ =450 nm).

7.3.3 Monomer degradation

To get insight into the chemical stability of the polymers, the degradation kinetics of the monomers under physiological conditions (pH 7.4, 37 °C) were studied. The samples were analyzed by analytical HPLC and the resulting natural logarithm of the monomer concentration was plotted against time to obtain the monomer half-life times ($t_{1/2}$).

Table 2: Half-life times ($t_{1/2}$) of the monomers at pH 7.4 and 37 °C.

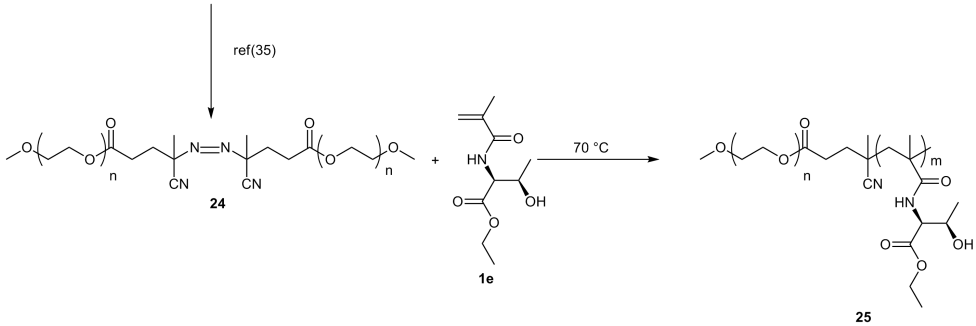
Monomer	Half-life times (days)[a]	Monomer	Half-life times (days)[a]
MA-Ser-OMe (**1a**)	5.0 ± 0.6	MA-Thr-OMe (**1d**)	5.4 ± 0.4
MA-Ser-OEt (**1b**)	11.0 ± 0.1	MA-Thr-OEt (**1e**)	11.8 ± 0.8
MA-Ser-O*i*Pr (**1c**)	40.5 ± 0.5	MA-Thr-O*i*Pr (**1f**)	33.7 ± 4.5

[a](average ± SD of two independent experiments)

Table 2 shows that MA-Ser-OMe and MA-Thr-OMe hydrolyzed most rapidly, followed by the ethyl and the isopropyl esters, respectively. These data also show that the monomer half-life times are only determined by the bulkiness of the esters, and are independent of the amino acid used. Interestingly, the methyl group on the threonine side chain does not interfere with the rate of hydrolysis (Table 2), while it clearly had an influence on the cloud point of the corresponding polymers. The methyl group on the threonine side chain makes the monomer more hydrophobic compared to the hydrogen atom on the serine side chain. For the ester hydrolysis the type of leaving group (ester) is the most important factor.

7.3.4 Thermoresponsive polymeric micelles

To study the possibilities of using the serine- and threonine-based methacrylate polymers for application as thermoresponsive polymeric micelles, a thermoresponsive amphiphilic block co-polymer was synthesized. Firstly, a $mPEG_{5000}$-substituted macroinitiator ($(mPEG_{5000})_2$ABCPA) was synthesized according to the procedure described by Neradović et al.[35] Poly(MA-Thr-OEt) was chosen as the thermosensitive block since it has a CP below body temperature (24 °C). Poly(MA-Thr-OEt)-b-PEG 5000 (entry 25) was synthesized by radical polymerization using MA-Thr-OEt as monomer and $(Peg5000)_2$ABCPA as radical initiator with a monomer/initiator (M/I) ratio of 100/1 (Scheme 2). The block co-polymer was obtained in a yield of 63%. According to NMR the polymers had an average of 30 repeating units of MA-Thr-OEt.



Scheme 2: Synthesis of poly(MA-Thr-OEt)-b-PEG 5000 co-polymer

Table 3: Characteristics of the ABCPA macroinitiator and the synthesized amphiphilic block co-polymer.

Entry	Block co-polymer	M_n[a] (kDa)	M_w[a] (kDa)	M_w/M_n	M_n[c] Thr-block (kDa)	Z_{ave}[d]	PD[d]	Yield (%)
24	$(Peg5000)_2$ABCPA	11.1	12.2	1.1	-	-	-	46
25	poly(MA-Thr-OEt)-b-PEG 5000	13.0	22.7	1.7	6.4	216	0.06	93

[a]M_n and M_w were determined by GPC with 10 mM LiCl in DMF as an eluent and PEG standards were used for calibration; [b];. M_n[c] of the MA-Thr-OEt block was determined by ^{1}H-NMR, [d] determined by DLS, 1 mg/mL dispersion in H_2O at 25 °C, particles were prepared via rapidly heating an ice-cold polymer solution to above its CP while stirring.

Dynamic light scattering (DLS) measurements showed that when a solution of poly(MA-Thr-OEt)-b-PEG 5000 in H_2O was heated from 0 °C to 25 °C, the clear solution became turbid at 22 °C (which resulted in a high particle count rate (Figure 7), indicating that particles have been formed. When the dispertion was cooled the particle count rate drops till there were no detectable particles at about 20 °C, indicating that the amphiphilic polymer has become hydrophilic again and dissolved completely. This critical micelle temperature (CMT) range (20 °C till 25 °C) corresponds with that of the homopolymeric CP of poly(MA-Thr-OEt) (24 °C).

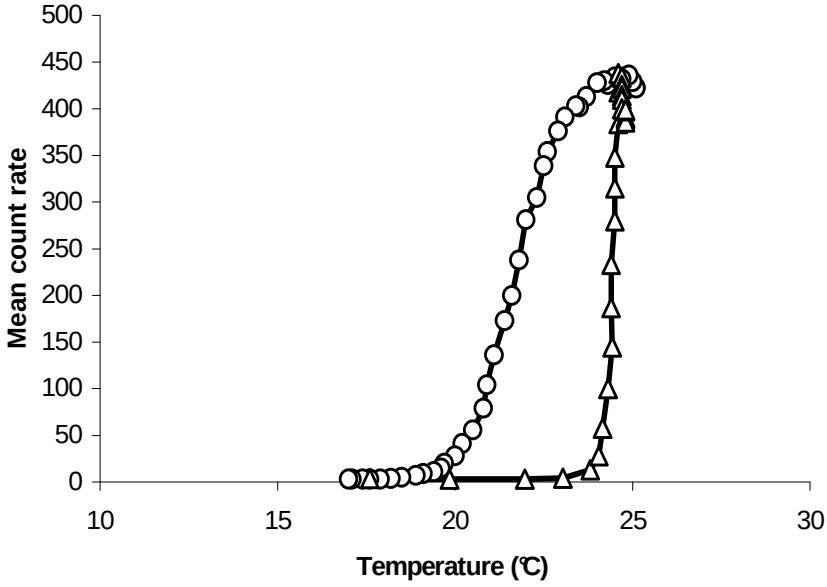


Figure 7: Mean count rate of poly(MA-Thr-OEt)-b-PEG 5000 dissolved in H_2O (1 mg/mL)(○)) from 17 °C to 25 °C (Δ) and from 25 °C to 17 °C (○).

The particles were incubated at 37 °C and at pH 5.0 and 7.4, and in time the scattering intensity was determined by DLS at 37 °C with a fixed attenuator setting. At pH 5.0, where the ester hydrolysis is minimal,[36] the mean count rate (MCR) was almost constant over 700 h at 37 °C, indicating that the particles are stable at pH 5.0 (Figure 8). Moreover, the diameter and PD of the particles remained

constant in time (Z_{ave}: 300 nm, PD: 0.1-0.15). In contrast, the mean count rate of the particles at pH 7.4 decreased in time, indicating that the ester bonds hydrolysed, resulting in hydrophilization of the micelles and ultimately destabilization.[12,37] This destabilization also resulted in an increase of PD from 0.1 at the start to 0.5 after 700 h, whereas the diameter of the particles remained constant (Z_{ave}: 300 nm). The stability of the particle diameter is unexpected and might the result of temperature fluctuations during the measurements. From Figure 8 can been seen that after approximately 400 h (16.5 days) the mean count rate was halved in relation to the starting value at pH 7.4.

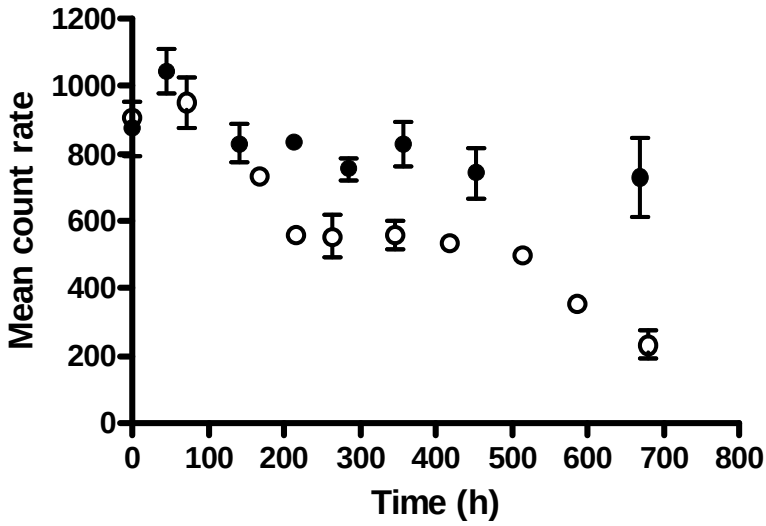


Figure 8: Stability of poly(MA-Thr-OEt)-b-PEG 5000 particles at 37 °C and at pH 7.4 (○) and pH 5.0 (●), as determined by dynamic light scattering. The data are shown as average ± SD, n = 3.

7.4 Conclusions

Six monomers were synthesized, based on the methyl, ethyl and isopropyl esters of N^{α}-methacryloyl-L-serine and L-threonine. With these six monomers homo-polymers as well as various co-polymers were synthesized. It was shown that these polymers had thermoreversible properties with a cloud point that ranges between 1.5 - 100 °C, depending on the monomer characteristics. The cloud point of the polymers could be tailored by varying the type of the ester moiety as well as the amino acid derivative. The LCST behavior of the polymers could be further fine-tuned by synthesizing co-polymers.

Monomer degradation studies showed that the methyl esters hydrolyzed more rapidly than ethyl ester followed by the isopropyl esters. These results indicated that besides the cloud point also the degradation kinetics of the polymers could be tailored.

The block co-polymer poly(MA-Thr-OEt)-b-PEG 5000 was synthesized, which was soluble in water below its CP and formed micelles above 21 °C. When the micelles were incubated at pH 7.4 at 37 °C they slowly destabilized due to the hydrolysis of the ester bonds present in the polymer. In conclusion, new types of amino acid based LCST polymers were synthesized with independently tailorable cloud points.

7.5 Materials and Methods

Materials and General Procedures. L-Serine and AIBN were purchased from Acros Organics. $SOCl_2$, L-threonine and methacryloyl chloride were purchased from Fluka. Peptide synthesis grade DMF, MeOH, EtOH, *i*PrOH and dioxane were obtained from Biosolve. Triethylamine was purchased from Merck. Solvents used for extractions and column chromatography were distilled prior to use. Thin layer chromatography (TLC) was performed on Merck silica gel 60 F-254 plates. Spots were visualized by UV light, ninhydrin, TDM/Cl_2,[38] $KMnO_4$. Silicycle silica 60 Å (particle size 41-63 μm) was used for column chromatography. ^{1}H-NMR spectra were recorded on a Varian Gemini 300 MHz spectrometer and chemical shifts are given in ppm (δ) relative to the internal reference TMS (0.00 ppm). ^{13}C-NMR spectra were recorded at 75.5 MHz and chemical shifts are given in ppm (δ) relative to either DMSO-d_6 or $CDCl_3$ (77.0 ppm). Analytical RP-HPLC runs were carried out on a Shimadzu automated HPLC system equipped with a UV/VIS detector operating at λ = 214 and 254 nm and using an Alltech Prosphere C18 column (250 × 4.6 mm, particle size: 5 μm, pore size: 300 Å) at a flow rate of 1 mL/min using a linear gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95:5 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 20 min.

Gel Permeation Chromatography. GPC was performed on a Waters 2695 Controller equipped with a refractive index detector (model 2414). The analyses were run on a PLgel MIXED-D column (particle size: 5 μm) (Polymer Laboratories) at 40 °C using 10 mM LiCl in DMF as the mobile phase at a flow rate of 0.7 mL/min. The polymers were dissolved in DMF (containing 10 mM LiCl) at a concentration of 5 mg/mL for at least 24 h before filtering them through a 0.45 μm filter prior analysis. The samples were analyzed and calibration was done using PEG as standards (M: 194-439,600 g/mol; Polymer Laboratories). Peak areas were determined with Empower Software Version 1154 (Waters Associates Inc.).

Cloud Point Determination. CP measurements were preformed on a Shimadzu UV-2450 UV/VIS spectrophotometer. Polymer sample solutions were prepared by dissolving 1 mg of polymer per mL H_2O. The samples were placed in a cuvette, heated from 0 °C to 95 °C and cooled to 0 °C while measuring the scattering at 450 nm at a heating/cooling rate of 1°C/min. The cloud point was determined by extrapolating the slope of the scattering increase to zero scattering.

Dynamic Light Scattering (DLS). DLS was performed on a Malvern CG-3 multi-angle goniometer (Malvern Ltd., Malvern, U.K.) equipped with a He-Ne JDS Uniphase laser (λ = 632.8 nm, 22 mW output power), an optical fiber based detector, a digital LV/LSE-5003 correlator and a temperature controller (Julabo Waterbath). Time correlation functions were analysed using the ALV-60X0 Software V.3.X provided by Malvern. Scattering of the dispersions was measured at an angle of 90° between 17 and 25 °C in an optical quality 8 mL borosilicate cell. A cell with approximately 1 mL micellar dispersion (1 mg/mL) was placed in the DLS machine and measured at regular time intervals within one heating/cooling cycle.

7.5.1 Monomer Synthesis

HCl·H-Ser-OMe (2a). To a cooled suspension (0 °C) of L-serine (5.25 g, 50 mmol) in MeOH (200 mL), $SOCl_2$ (18 mL, 250 mmol) was added drop wise. After the addition of $SOCl_2$, the cooling bath was removed and the reaction mixture was stirred for 16 h at room temperature. Subsequently, the solvent was removed in vacuo, and the residue was coevaporated with toluene (3×) and chloroform (3×) to remove any $SOCl_2$. The residual oil was triturated with diethyl ether, and crystals were formed. These crystals were filtered off, washed with diethyl ether and dried. Methyl ester **2a** was obtained as a white solid in 84% yield (6.6 g). R_f = 0.87 ($CHCl_3$/MeOH/25% NH_4OH 8:4:1.5 v/v/v). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.74 (s, 3H, OCH_3), 3.83 (s, 2H, H^β), 4.09 (m, 1H, H^α), 5.64 (br s, 1H, OH), 8.60 (br s, 3H, NH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 53.1, 54.3, 59.8, 168.8.

HCl·H-Ser-OEt (2b). According to the procedure (50 mmol scale) as described for HCl·H-Ser-OMe (**2a**). HCl·H-Ser-OEt (**2b**) was obtained as a white crystalline solid in 98% yield (8.3 g). R_f = 0.87 ($CHCl_3$/MeOH/25% NH_4OH 8:4:1.5 v/v/v). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.24 (t (*J* 7.0 Hz), 3H, CH_3), 3.83 (s, 2H, H^β), 4.07 (t (*J* 3.4 Hz), 1H, H^α), 4.2 (m, OCH_2), 5.62 (br s, 1H, OH), 8.55 (br s, 3H, NH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 13.9, 54.3, 59.5, 61.7, 168.0.

HCl·H-Ser-O*i*Pr (2c). According to the procedure (50 mmol scale) as described for HCl·H-Ser-OMe (**2a**). HCl·H-Ser-O*i*Pr **(2c)** was obtained as a white crystalline solid in 80% yield (7.4 g). R_f = 0.89 ($CHCl_3$/MeOH/25% NH_4OH 8:4:1.5 v/v/v). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.24 (d (*J* 2.8 Hz),

6H, CH_3), 3.81 (s, 2H, H^{β}), 4.02 (m, 1H, H^{α}), 4.99 (m, 1H, OCH), 5.61 (br s, 1H, OH), 8.52 (br s, 3H, NH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 21.4, 54.4, 59.5, 69.5, 167.5.

HCl·H-Thr-OMe (2d). To a cooled suspension (0 °C) of L-threonine (5.96 g, 50 mmol) in MeOH (200 mL), $SOCl_2$ (18 mL, 250 mmol) was added drop wise. After the addition of $SOCl_2$, the cooling bath was removed and the reaction mixture was heated under reflux for 6 h. Subsequently, the solvent was removed in vacuo, and the residue was coevaporated with toluene (3×) and chloroform (3×) and dried in vacuo. HCl·H-Thr-OMe **(2d)** was obtained as colorless oil in 99% yield (8.5 g). R_f = 0.92 ($CHCl_3$/MeOH/25% NH_4OH 8:4:1.5 v/v/v). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.20 (d (*J* 6.3 Hz), 3H, H^{γ}), 3.75 (s, 3H, OCH_3), 3.93 (d (*J* 3.9 Hz), 1H, H^{β}), 4.13 (m, 1H, H^{α}), 5.65 (br d (*J* 5.0 Hz), 1H, OH), 8.40 (br s, 3H, NH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 20.0, 52.7, 57.9, 65.0, 168.7.

HCl·H-Thr-OEt (2e). According to the procedure (50 mmol scale) as described for HCl·H-Thr-OMe (**2d**). HCl·H-Thr-OEt **(2e)** was obtained as slightly yellow oil in 92% yield (8.4 g). R_f = 0.88 ($CHCl_3$/MeOH/25% NH_4OH 8:4:1.5 v/v/v). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.21 (d (*J* 6.6 Hz), 3H, H^{γ}), 1.24 (t (*J* 7.0 Hz), 3H, CH_3), 3.90 (m, 1H, H^{α}), 4.13 (m, 1H, H^{β}), 4.21 (q (*J* 7.2 Hz), 2H, OCH_2), 8.39 (br s, 3H, NH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 13.9, 20.0, 57.8, 61.7, 65.0, 168.1.

HCl·H-Thr-O*i*Pr (2f). According to the procedure (50 mmol scale) as described for HCl·H-Thr-OMe **(2d)**. HCl·H-Thr-O*i*Pr **(2f)** was obtained as yellow oil in 99% yield (9.8 g). R_f = 0.92 ($CHCl_3$/MeOH/25% NH_4OH 8:4:1.5 v/v/v). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.26 (m, 9H, $CH(C\underline{H}_3)_2$ (6H), H^{γ} (3H)), 3.83 (d (*J* 4.13), 1H, H^{α}), 4.11 (br s, 1H, H^{β}), 5.01 (m, 1H, OCH), 5.64 (br d (J 4.4 Hz), 1H, OH), 8.35 (br s, 3H, NH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 20.1, 21.3, 21.5, 57.9, 65.2, 69.5, 167.6.

***N*$^{\alpha}$-Methacryloyl-Ser-OMe (1a).** HCl·H-Ser-OMe **(2a)** (6.7 g, 43 mmol) was dissolved in dioxane/water 1:1 v/v (300 mL) and this solution was cooled on ice before methacryloyl chloride (4.2 mL, 43 mmol) and TEA (18 mL, 129 mmol) were added drop-wise at pH 8 to 9. After the addition was complete, the cooling bath was removed and the reaction mixture was stirred 16 h at room temperature. Then, the solvents were removed in vacuo and the residue was redissolved in Et_2O and the remaining solid was removed by filtration and washed with Et_2O. The filtrate was concentrated in vacuo and the obtained residue was purified by column chromatography, in first instance with hexane/Et_2O 1:1 v/v as eluent to remove radical inhibitor 4-methoxyphenol (MEHQ) followed by Et_2O. *N*$^{\alpha}$-Methacryloyl-Ser-OMe **(1a)** was obtained as colorless oil in 33% yield (2.5 g). R_f = 0.19 (Et_2O). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.99 (s, 3H, CH_3), 3.05 (br s, 1H, OH), 3.80 (s, 3H, OCH_3),

3.96 (m, 2H, H^{β}), 4.71 (m, 1H, CH^{α}), 5.42 (s, 2H, H_2C=C (1H)), 5.81 (s, 2H, H_2C=C (1H)), 6.82 (br d (*J* 6.3 Hz), 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 18.7, 52.9, 55.1, 62.8, 121.3, 139.4, 169.3, 171.4.

***N*$^{\alpha}$-Methacryloyl-Ser-OEt (1b).** According to the procedure (49 mmol, HCl·H-Ser-OEt **(2b)**) as described for ***N*$^{\alpha}$-**methacryloyl-Ser-OMe **(1a)**. *N*$^{\alpha}$-Methacryloyl-Ser-OEt **(1b)** was obtained as a white solid in 47% yield (4.5 g). R_f = 0.31 (Et_2O). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.31 (t (*J* 7.0), 3H, CH_2CH_3), 1.99 (s, 3H, CH_3), 3.98 (m, 2H, H^{β}), 4.26 (q (*J* 6.5), 2H, OCH_2), 4.69 (m, 1H, H^{α}), 5.42 (s, 1H, H_2C=C (1H)), 5.82 (s, 1H, H_2C=C (1H)), 6.84 (br d (*J* 6.1 Hz), 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 13.7, 18.0, 54.5, 61.4, 62.4, 120.5, 138.8, 168.4, 170.2.

***N*$^{\alpha}$-Methacryloyl-Ser-O*i*Pr (1c)**. According to the procedure (40 mmol, HCl·H-Ser-O*i*Pr **(2c)**) as described for ***N*$^{\alpha}$-**methacryloyl-Ser-OMe **(1a)**. *N*$^{\alpha}$-Methacryloyl-Ser-O*i*Pr **(1c)** was obtained as a viscous oil in 75% yield (6.5 g, 30 mmol). R_f = 0.47 (Et_2O). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.89 (dd (J_{ax} 2.20 Hz J_{bx} 6.33 Hz), 6H, CH ($C\underline{H}_3)_2$), 2.00 (m, 3H, $C\underline{H}_3$C=CH), 3.04 (br s, 1H, OH), 3.97 (d (*J* 3.9 Hz), 2H, CH_2), 4.64 (m, 1H, H^{α}), 5.10 (sept (*J* 6.3 Hz), 1H, OCH), 5.41 (s, 1H, H_2C=C (1H)), 5.82 (s, 1H, H_2C=C), 6.82 (br d (*J* 6.1 Hz), 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 18.7, 21.9, 55.4, 63.9, 70.1, 121.0, 139.4, 168.9, 170.2.

***N*$^{\alpha}$-Methacryloyl-Thr-OMe (1d).** According to the procedure (52 mmol, HCl·H-Thr-OMe **(2d)**) as described for *N*$^{\alpha}$-methacryloyl-Ser-OMe **(1a)**. *N*$^{\alpha}$-Methacryloyl-Thr-OMe **(1d)** was obtained as a viscous oil in 88 % yield (9.2 g, 46 mmol). R_f = 0.28 (Et_2O). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.24 (d (*J* 6.6 Hz), 3H, H^{γ}), 2.01 (t (*J* 1.2 Hz), 3H, CH_3C=C), 2.29 (br s, 1H, OH), 3.79 (s, 3H, OCH_3), 4.39 (m, 1H, H^{β}), 4.66 (m, 1H, H^{α}), 5.42 (s, 1H, H_2C=C (1H)), 5.81 (s, 1H, H_2C=C (1H)), 6.60 (br d, 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 18.8, 20.2, 52.9, 57.5, 68.3, 120.8, 139.7, 169.0, 171.8.

***N*$^{\alpha}$-Methacryloyl-Thr-OEt (1e).** According to the procedure (46 mmol, HCl·H-Thr-OEt **(2e)**) as described for *N*$^{\alpha}$-methacryloyl-Ser-OMe **(1a)**. *N*$^{\alpha}$-Methacryloyl-Thr-OEt **(1e)** was obtained as a colorless oil in 96 % yield (9.5 g, 44 mmol). R_f = 0.47 (Et_2O). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.26 (m, 6H, $OCH_2C\underline{H}_3$ (3H), H^{γ} (3)), 2.01 (t (*J* 0.8 Hz), 3H, CH_3), 2.24 (br s, 1H, OH), 4.24 (q (*J* 7.2 Hz), 2H, OCH_2), 4.37 (br d (*J* 3.0 Hz), 1H, H^{α}), 4.64 (m, 1H, H^{β}), 5.41 (s, 1H, H_2C=C (1H)), 5.80 (s, 1H, H_2C=C (1H)), 6.57 (br d (*J* 7.7 Hz), 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 14.0, 18.5, 20.0, 57.7, 61.5, 67.7, 120.5, 139.4, 169.2, 170.9.

***N*$^{\alpha}$-Methacryloyl-Thr-O*i*Pr (1f).** According to the procedure (52 mmol, HCl·H-Thr-O*i*Pr **(2f)**) as described for *N*$^{\alpha}$-methacryloyl-Ser-OMe **(1a)**. *N*$^{\alpha}$-Methacryloyl-Thr-O*i*Pr **(1f)** was obtained as a

white solid in 79 % yield (9.3 g, 41 mmol). R_f = 0.63 (Et_2O). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.26 (m, 9H, $CH(C\underline{H}_3)_2$ (6H), H^γ (3H)), 2.01 (m, 3H, CH_3), 2.12 (br s, 1H, OH), 4.36 (m, 1H, H^α), 4.62 (m, 1H, H^β), 5.10 (septet (*J* 6.2 Hz), 1H, $C\underline{H}(CH_3)_2$), 5.41 (s, 1H, H_2C=C (1H)), 5.80 (s, 1H, H_2C=C (1H)), 6.55 (br d (*J* 8.0 Hz), 1H, NH,). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 18.7, 20.2, 21.8, 57.8, 68.1, 69.5, 120.5, 139.6, 169.2, 170.6.

7.5.2 Polymer synthesis

Typical homopolymer synthesis. Monomer **1a - f** (0.5 g) and AIBN (the monomer/initiator (M/I) ratio was 100/1) were dissolved in dry DMF (5 mL) and degassed by three freeze-pump-thaw cycles. The reaction mixture was stirred for 48 h at 70 °C after which the solvent was removed in vacuo and the residue was redissolved in MeOH (5 mL) and precipitated in Et_2O (40 mL) under vigorous stirring. The precipitate was collected by centrifugation (3000 rpm, 5 min) and the solvent was decanted. After drying in vacuo, the polymers were obtained as white solids in a general yield of 82 to 96 %.

Typical synthesis of co-polymers (1:1 co-monomer ratio). To an equimolar mixture of co-monomer 1 and co-monomer 2 (1.5 mmol) AIBN (5 mg, 30 μmol) was added and subsequently dissolved in dry DMF (5 mL) and the solution was degassed by three freeze-pump-thaw cycles. The reaction mixture was stirred for 48 h at 70 °C after which the solvent was removed in vacuo and the residue was redissolved in chloroform (5 mL) and precipitated in hexane (40 mL) under vigorous stirring. The precipitate was collected by centrifugation (3000 rpm, 5 min) and the solvent was decanted. After drying in vacuo, the co-polymers were obtained as white solids in a yield of 32 to 95 %.

7.5.3 Degradation experiments

Monomer degradation. Monomer **1a - f** (15 mg) was dissolved in 100 mM sodium phosphate buffer pH 7.4 (5 mL) and incubated at 37 °C. The pH was measured before and after the degradation at 37 ˚C. At regular time intervals a sample (100 μL) was drawn, quenched with ice-cold 1 M Na-acetate buffer pH 3.8 (200 μL) and stored at 4˚C prior to HPLC analysis.

7.5.4 Synthesis of thermoresponsive amphiphilic block co-polymer.

Synthesis of (PEG monomethyl ether 5000)$_2$-ABCPA (3). To a solution of 4,4-azobis(4-cyanopentanoic acid) (ABCPA) (558 mg, 2 mmol), 4-(dimethylamino)pyridinium 4-tosylate (DPTS)

(0.39 g, 1.3 mmol) and DCC (1.36 g, 6.6 mmol) in THF/DCM (320 mL v/v 1:1) PEG monomethyl ether Mw 5000 (20 g, 4 mmol) was added. The obtained reaction mixture was stirred for 48 h at room temperature. The suspension was filtered through a plug of Celite and the filtrate was concentrated in vacuo. The residue was redissolved in H_2O/CH_3CN and lyophilized. (PEG monomethyl ether 5000)$_2$-ABCPA (**3**) was obtained as a white solid in a 46% yield (9.46 g). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.70 (d, 6H, CH_3), 3.80 (m, 900H, CH_2).

Synthesis of poly(MA-Thr-OEt)-b-PEG 5000 co-polymer. MA-Thr-OEt (493 mg, 2.30 mmol) and (PEG monomethyl ether 5000)$_2$-ABCPA **(3)** (238 mg 0.023 mmol) were dissolved in degassed DMF and stirred for 72 h at 70 °C. Subsequently, the solvent was removed under reduced pressure and the residue was redissolved in chloroform (5 mL). This solution was precipitated in hexane (40 mL) under vigorous stirring. The precipitate was collected by centrifugation (3000 rpm, 5 min) and the solvent was decanted. After drying in vacuo, the co-polymer was obtained as white solids in a yield of 63% (0.46 g).

7.5.5 Particle preparation and destabilization of Poly(MA-Thr-OEt)-b-PEG micelles.

Particle preparation and destabilization. Poly(MA-Thr-OEt)-b-PEG 5000 co-polymer (3 mg) was dissolved in ice-cold 100 mM phosphate buffer (3 mL, pH 7.4) containing 0.02% NaN_3 (to prevent bacteria growth). The polymer solution was rapidly heated with a water-bath, while shaken, till a temperature above CP was reached. Subsequently, the polymer solution was incubated at 37 °C and particle size, mean count rate and PDI were determined at regular time intervals by DLS at 37 °C.

7.6 References

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Chapter 8

Summary and Perspectives

This thesis deals with the synthesis and characterization of peptide-based polymer-constructs that can be applied for a variety of purposes such as drug delivery systems, scaffolds for tissue-engineering and -repair, and as novel biomaterials.

8.1 Summary

Chapter 1 gives a general overview of peptide-based polymers, their synthesis and physico-chemical properties, the methods of characterization, and the range of (future) applications, and describes the current state-of-the-art of the Cu(I)-based cycloaddition reaction for the synthesis of peptide-based polymers, which forms the heart of this thesis. **Chapter 2** reviews the synthesis and application of biomedical and pharmaceutical polymers which have been synthesized via two "click" reactions, namely the copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) and the thiol-ene coupling (TEC) reaction. Both click reactions have been extensively used for the synthesis and chemo-selective conjugation of many new polymer constructs. However, both the CuAAC- as well as the TEC reaction still have some flaws e.g. cytotoxicity of the copper catalyst, and the susceptibility of the thiol functionality for oxidizing reagents. Nevertheless, the CuAAC- and TEC "click" reaction have acquired a central position in the synthesis as well as selective conjugation of polymers for biomedical and pharmaceutical applications, and it is expected that their importance for the design and synthesis of such polymers will increase rapidly in the coming years. In **chapter 3**, the model dipeptide azido-phenylalanyl-alanyl-propargyl amide has been designed and synthesized and initial microwave-assisted "click" polymerization reaction have been performed to get insight into the scope of this novel approach for the synthesis of peptide-triazole containing polymers as protein mimics. Depending on the reaction conditions, it was found that the outcome of the "click" reaction could be directed either toward large linear polymers containing up to 300 amino acid residues (Mn approx. 45,000 Da) or toward medium-sized peptide macrocycles containing 4 to 20 amino acid residues (cyclic dimer to decamer). To prove the generality of this approach, a second and more functionalized monomer consisting of N_3-β-Ala-Pro-Gly-Ser-propargyl amide, which represents the repetitive sequence within spider silk, was synthesized and polymerized. It was found that the reaction conditions as defined with the model dipeptide could be applied for this tetrapeptide to obtain either linear or cyclic peptide-based polymers/oligomers. As a further extension of the "click"-based polymerization reaction, **chapter 4** describes the design and synthesis of two novel *biodegradable* monomers, azido-phenylalanyl-alanyl-lysyl-propargyl amide and azido-phenylalanyl-alanyl-glycoloyl-lysyl-propargyl amide. The polymers that were obtained from these two monomers contained 33 to 100 amino acid residues per polymer chain (Mn between 4,500 – 13,900 Da). The newly synthesized polymers could be hydrolyzed by the proteases trypsin and chymotrypsin into

well-defined fragments. Since the polymers, derived from azido-phenylalanyl-alanyl-glycoloyl-lysyl-propargyl amide, contain ester bonds it was shown that these polymers were also sensitive toward chemical hydrolysis in a pH-dependent manner. In **chapter 5** the CuAAC reaction was used to synthesize enzymatically degradable PEG-based hydrogels, based on alkyne-functionalized star-shaped PEG derivatives and a protease-sensitive peptide sequence functionalized with two azide moieties. The synthesized peptide-based hydrogels had fully elastic properties, and the storage modulus (G') of the hydrogels could be tailored by varying the solid content, the type of the PEG molecules, and by variation of the azide/alkyne ratio. The gelation time of the hydrogels showed to be dependent on the solid content of the hydrogels. Incubation of the hydrogels with trypsin resulted in a complete degradation of the hydrogels within a few hours. In **chapter 6** the microwave-assisted CuAAC reaction was utilized for the synthesis of small cyclic oligomers based on the amyloidogenic Aβ(16-22) peptide sequence. This peptide was chosen since its strong tendency to form antiparallel β-sheets that ensured the formation of intermolecular hydrogen bridges on which the supramolecular assembly of the individual cyclic oligomers was based. Gelation behavior and the self-assembly of the linear monomer and the cyclic monomer and dimer were studied. Significant differences were observed in the morphology of the supramolecular aggregates of these three peptides that could be explained by alterations of the hydrogen bond network. In **chapter 7** thermoresponsive polymers based on N^{α}-methacryloyl-functionalized serine- and threonine esters were synthesized by free radical polymerization. The lower critical solution temperature (LCST) of the polymers could be tailored by varying the type of the ester moiety as well as the amino acid derivative. The LCST behavior of the polymers could be fine-tuned by the synthesis of co-polymers. It was shown that the ester bonds within the polymers were sensitive for chemical hydrolysis and their (partial) cleavage resulted in an increase in the LCST. Furthermore, it was also shown, that by the synthesis of a block-co-polymer with PEG, thermoresponsive micelles could be obtained. Hydrolysis of the ester bonds resulted in a destabilization of these micelles; a possible implication of controlled-release applications of this type of block-co-polymers.

8.2 Perspectives

In this thesis several peptide-based polymers have been synthesized via the CuAAC reaction based on unprotected peptide sequences to obtain well-defined peptide-based polymers with tailored properties.

Non-viral gene delivery

Inspired by the work of Reineke and coworkers[1,2] and the work of Dervan and coworkers, who showed that macromolecules that contain various heterocyclic residues, such as derivatives of pyrrole

and imidazole are able to bind nucleic acids [3] The two biodegradable polymers as described in chapter 4, azido-phenylalanyl-alanyl-lysyl-propargyl amide (N_3-Phe-Ala-Lys-propargyl amide) and azido-phenylalanyl-alanyl-glycoloyl-lysyl-propargyl amide (N_3-Phe-Ala-Glyc-Lys-propargyl-amide) were tested for their ability to act as a carrier for non-viral gene delivery. The polymers were examined for the ability to bind DNA using the agarose gel electrophoretic shift assay. The polymers were mixed with DNA at various polymer-Nitrogen to plasmid-Phosphate (N/P) ratios, loaded onto the gel and electrophoresed. Poly(N_3-Phe-Ala-Lys-propargyl amide) (M_w 25,600 Da, polymer entry 5, Table 1, chapter 4) was able to condense plasmid DNA at a N/P ratios > 5, while poly(N_3-Phe-Ala-Glyc-Lys-propargyl amide) (M_w 14,000, polymer entry 10, Table 1, chapter 4) was able to form polyplexes at N/P ratios above 20.

Based on these data it was investigated to which extent the polymers were efficient transfectants or induce undesired cytotoxicity effects. Unfortunately, the tested polymers did not show any transfection. However, polyplexes of poly(N_3-Phe-Ala-Lys-propargyl amide) and poly(N_3-Phe-Ala-Glyc-Lys-propargyl) amide had a significant lower toxicity than poly(L-lysine). Although promising with respect to toxicity issues, transfection efficiency has to be optimized before these polymers can be used as efficient non-viral gene delivery systems.

Cyclic oligomers

Cyclic peptides have received great interest over the past decades since they play an important role in Nature. Furthermore cyclic peptides are often more stable *in vivo* and have a reduced conformational freedom compared to their linear counterparts, therefore, they have proven to be potential drug candidates.[4] However, not much research has been done regarding the role of cyclic oligomers, due to difficulties with the synthesis and isolation. In chapter 3 and 6 it was shown that the microwave assisted CuAAC reaction can be employed for the synthesis of various cyclic oligomers. These cyclic oligomers might play a role as antimicrobial-, antifungal agent[5] or in ion chelation and transport *in vivo*.[6,7]

Cu-free click chemistry

The CuAAC reaction has been successfully employed for the synthesis of peptide-based polymers. However the CuAAC reaction suffers from one major drawback: the need for a cytotoxic copper catalyst. Especially for polymers with applications in the pharmaceutical and biomedical field, the use of a copper catalyst might result in adverse effects. As an alternative for Cu(I) as catalyst to activate acetylenes, the use of ring strain has been investigated. However, the first generation of cyclooctynes was hampered with a relatively low reactivity towards azides, with respect to the CuAAC reaction, resulting in long reaction times and lower coupling efficiencies. Furthermore the first generation of

cyclooctynes suffered from poor water solubility.[8,9] Recently a second generation of difluorinated cyclooctynes was reported, and these difluorinated cyclooctynes possess similar reaction kinetics as the Cu(I)-catalyzed and have improved water solubility, however,their synthesis is rather difficult.[10-12]

The hydrogel system described in chapter 5 might benefit from the development of new generations of copper-free click reactions. It might be possible that residual copper traces were responsible for the inactivation of the protease plasmin.
Furthermore, by introducing a copper-free click system it is possible to synthesise injectable hydrogels. In this case the polymer system can be injected as a two component system into the human body. Due to the body temperature the gel can be generated *in situ* within minutes.

LCST
The thermoresponsive micelles described in chapter 7 can be used for encapsulation of hydrophobic drugs. Ideally, the loaded micelles can accumulate in a targeted tissue (e.g. tumor). Because of the slightly decreased pH value in the tumor, the ester bonds of the thermoresponsive block will degrade and thereby releasing the drug in the targeted tissue.

In this thesis model peptides were used to develop new methodologies for the synthesis of peptide-based polymers. These model peptides were used to optimise the reaction conditions. The optimised reaction conditions can be applied for the synthesis of biologically relevant peptide-based biopolymers. Recently Guan and co-workers reported on the synthesis of peptide-based polymers that can fold into well-defined β-sheets followed by a self-assembly into hierarchical nanostructures based on our approach as described in this thesis.[13]

8.3 References

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Appendices

Nederlandse Samenvatting

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Dankwoord

Nederlandse Samenvatting

Polymeren met repeterende aminozuursequenties komen veelvuldig in de natuur voor. Bekende voorbeelden van dit type polymeren zijn spinnenzijde, mossellijm, collageen en antivries-eiwitten. Deze klasse van polymeren hebben unieke eigenschappen die gebruikt kunnen worden voor farmaceutische en biomedische toepassingen. Voor deze toepassingen is het belangrijk dat deze polymeren biodegradeerbaar zijn, hetzij via enzymatische hydrolyse, hetzij door spontane hydrolyse als functie van de zuurgraad van het milieu waarin deze polymeren zich bevinden. Deze biodegradeerbaarheid is van belang, zodat daarmee de kans op toxische effecten klein is. Helaas is het met de huidige technieken niet mogelijk om deze polymeren in grote hoeveelheden uit de natuurlijke matrix te isoleren. Daarnaast is er sterke behoefte deze polymeren chemisch te modificeren zodat ze breder toepasbaar zijn in farmaceutische applicaties. Daarom heeft men inmiddels verschillende methoden ontwikkeld om polymeren met repeterende aminozuur sequenties te synthetiseren. Echter deze methoden hebben verschillende nadelen en het is daarom van belang nieuwe syntheseroutes te ontwikkelen.

De meest toegepaste methode voor het synthetiseren van polymeren met repeterende aminozuur sequenties maakt gebruik van peptiden die via de N-terminus gekoppeld worden aan een polymeriseerbare groep. Op deze manier ontstaat er een polymeer met een synthetische hoofdketen met het peptide in de zijketens. Door de synthetische hoofdketen zijn deze polymeren niet biodegradeerbaar en kunnen daarom toxisch zijn. Het is echter ook zo dat synthesemethoden, waarbij de peptiden wel onderdeel van de hoofdketen zijn, gebruik maken van instabiele beginstoffen, het gebruik van beschermgroepen vereisen (met als nadeel dat het volledig verwijderen van deze beschermgroepen na polymerisatie kan soms problematisch zijn) en bovendien beperkt zijn in de toepasbare aminozuurresidue(n).

Dit proefschrift beschrijft een nieuwe methode om polymeren met repeterende aminozuursequenties the synthetiseren die deze beperkingen niet kent. Deze polymerisatiemethode is gebaseerd op de koper(I)-gekatalyseerde 1,3-dipolaire cycloadditiereactie tussen een alkyn en een azide, waarbij een triazool ring wordt gevormd, als mimeticum voor een natuurlijke peptide binding. De reactie heeft een groot aantal voordelen: de reactie verloopt snel, de reactie vindt plaats onder milde condities (lage temperatuur en waterige oplossingen) en de beginstoffen zijn stabiel en bovendien synthetisch eenvoudig toegankelijk. Daarnaast is de reactie ongevoelig voor andere functionele groepen die in peptiden voorkomen, waardoor het niet nodig is om de functionele groepen in de peptiden te beschermen.

In **hoofdstuk 1** wordt een beknopt overzicht gegeven van de huidige methodologie die gebruikt wordt voor de synthese van op peptide-gebaseerde polymeren. Tevens wordt een overzicht gegeven van mogelijke “click” reacties die gebruikt kunnen worden voor de synthese van dit type polymeren. Tenslotte wordt het doel van het promotieonderzoek beschreven en kort de resultaten besproken.

In **hoofdstuk 2** worden twee typen “click” reacties; namelijk de Michael additie tussen een thiol en alkeen en de Cu(I)-gekatalyseerde 1,3-dipolaire cycloadditiereactie tussen een alkyn en een azide uitvoerig beschreven. Hoofdstuk 2 is gebaseerd op een uitgebreid literatuuroverzicht van verschillende polymeersystemen met name gericht op farmaceutische en biomedische toepassingen die met behulp van deze “click” reacties zijn gesynthetiseerd.

In de hoofdstukken 1 en 2 wordt de huidige stand van zaken weergegeven. Vanaf hoofdstuk 3 wordt het eigen onderzoek beschreven.

Hoofdstuk 3 beschrijft de synthese en karakterisatie van een model-dipeptide, gefuctionaliseerd met een azide- en een alkyn groep. Dit modelpeptide wordt vervolgens gepolymeriseerd onder verschillende reactiecondities. De gevormde polymeren, worden gekarakteriseerd met verschillende analyse technieken. Hieruit bleek, dat afhankelijk van de polymerisatiecondities, de polymerisatie gestuurd kon worden in de richting van lange lineaire ketens (300 aminozuren in de keten) of tot het verkrijgen van korte cyclische structuren (4 tot 20 aminozuren in de keten). De grootte van de polymeren kon gestuurd worden door de monomeer concentratie te veranderen, gebruik van verschillende koper katalysatoren en de temperatuur. Daarnaast bleek dat het gebruik van magnetronverwarming als reactieparameter tijdens de polymerisatie niet alleen invloed heeft op de ketenlengte, maar ook zorgt voor verkorting van de reactietijd. Deze inzichten werden vervolgens getoetst op een ander peptide afgeleid van spinnenzijde. Ook met deze sequentie bleken de parameters van dezelfde invloed te zijn op de polymeerketens.

In **hoofdstuk 4** worden de synthese en karakterisatie van twee nieuwe monomeren, bestaande uit een tripeptide en een tetrapeptide, beschreven. De monomeren zijn zo ontworpen dat deze gevoelig zijn voor de enzymen trypsine en chymotrypsine. Omdat het tetrapeptide een esterband bevat, is dit monomeer ook hydrolyseerbaar als functie van de pH in waterig milieu. De monomeren worden gepolymeriseerd met behulp van de Cu(I)-gekatalyseerde 1,3-dipolairecycloadditie reactie onder invloed van magnetronverwarming. De ontstane polymeren werden vervolgens behandeld met de enzymen trypsine en chymotrypsine. Met de ninhydrine kleurentest en MALDI-TOF spectrometrie werd aangetoond dat beide polymeren gevoelig waren voor deze enzymen. In het geval waarbij het polymeer van het repeterende tripeptide werd afgebroken door trypsine bleek na 75 uur maar één, goed gedefinieerd, degradatieproduct aanwezig te zijn.

In **hoofdstuk 5** worden de synthese en karakterisatie beschreven van enzymatisch degradeerbare hydrogelen, welke gesynthetiseerd werden met behulp van de Cu(I)-gekatalyseerde 1,3-dipolaire

cycloadditiereactie. Hierbij werd een stervormig alkyn-gefunctionaliseerd polyethyleneglycol (PEG) molecuul gecrosslinked met een peptide uitgerust met twee azide-eenheden. De rheologische eigenschappen en de zwelling parameters van de hydrogelen konden gevarieerd worden door de concentraties van de bouwstenen, en door de ratio PEG/peptide te varieren, alsmede door de architectuur van de PEG derivaten te veranderen. Het peptide dat gebruikt is in de synthese van de hydrogelen is gevoelig voor de enzymen plasmine en trypsine. Echter, onder de geteste condities waren de hydrogelen ongevoelig voor plasmine, maar werden ze volledig gedegradeerd door trypsine.

In **hoofdstuk 6** wordt de synthese van het N-azido/C-alkynyl Aβ(16-22) peptide beschreven. Om cyclische Aβ(16-22) oligomeren te verkrijgen werd dit peptide gepolymeriseerd met behulp van de Cu(I)-gekatalyseerde 1,3-dipolaire cycloadditiereactie onder invloed van magnetronverwarming. Het cyclische monomer en cyclische dimeer, die werden geïsoleerd met behulp van HPLC, werden onderzocht op zelfassemblage-gedrag. De verschillen in zelfassemblage kwamen het sterkst tot uitdrukking in de supramoleculaire morfologieën die varieerden van lint-achtige structuren tot amyloïd-gelijkende fibrillen.

In **hoofdstuk 7** wordt de synthese van polymeren, gebaseerd op de methacrylamide esters van serine en threonine, beschreven. De monomeren werden gepolymeriseerd via een vrije radicaal polymerisatie. De gesynthetiseerde polymeren hadden een fase-overgangstemperatuur die afhankelijk was van het type aminozuur en van het type ester. Door de synthese van co-polymeren kon de fase-overgangstemperatuur gestuurd worden. Aangetoond werd dat de esterbindingen in de zijketens van de polymeren gevoelig waren voor chemische hydrolyse. De snelheid van de chemische hydrolyse was afhankelijk van het type ester. Door de hydrolyse van de esterbindingen werden carbonzuren gevormd. Hierdoor nam de hydrofiliciteit van het polymeer toe en steeg ook de fase-overgangstemperatuur. Dit werd geïllustreerd door de synthese van een blok-co-polymeer, opgebouwd uit een hydrofiel PEG-deel en een methacrylamide blok met temperatuurgevoelige eigenschappen. Bij een temperatuur lager dan de fase-overgangstemperatuur is dit polymeer wateroplosbaar, maar bij verwarming boven de fase-overgangstemperatuur, is het polymeer niet meer volledig wateroplosbaar, zodat deeltjes gevormd worden die gemeten konden worden met behulp van lichtverstrooiing. Wanneer de esterbindingen worden gehydrolyseerd, en daarmee de hydrofiliciteit van het polymeer toeneemt, destabiliseren de deeltjes en zal het aantal deeltjes in de loop van de tijd afnemen.

In **hoofdstuk 8** wordt de inhoud van dit proefschrift samengevat. Daarnaast worden er voorstellen gedaan voor mogelijke toepassingen en verbeteringen voor de in dit proefschrift beschreven polymeersystemen en synthese routes.

List of Abbreviations

Abbreviations of amino acids

Xxx	X	Amino acid
Ala	A	alanine
Gly	G	glycine
Glu	E	glutamic acid
Leu	L	leucine
Lys	K	lysine
Phe	F	phenylalanine
Pro	P	proline
Ser	S	serine
Thr	T	threonine
Val	V	valine

ABCPA	4,4-azobis(4-cyanopentanoic acid)
AIBN	2,2'-azobisisobutyronitrile
ATP	attached proton test
Boc	tert-butyloxycarbonyl
BOP	benzotriazol-1-yl-oxytris-dimethylamino-phosphonium hexafluorophosphate
Bzl	benzyl
CP	cloud point
CuAAC	copper(I)-catalyzed alkyne-azide cycloaddition
CuOAc	copper(I) acetate
Δ	chemical shift
d	doublet
Da	dalton
DCC	N,N'-dicyclohexylcarbodiimide
dd	double doublet
DCM	dichloromethane
Dipea	N,N’-diisopropylethylamine
DLS	dynamic light scattering
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DPTS	4-(dimethylamino)pyridinium 4-tosylate
ESI-MS	electro spray ionisation mass spectrometry
EtOAc	ethylacetate
Fmoc	9-fluorenylmethyl-oxycarbonyl
FTIR	fourier transform infrared spectroscopy
Glyc	glycolic acid
GPC	gel permeation chromatography
h	hour
HBTU	2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOBt	N-hydroxybenzotriazole
Hz	hertz
J	coupling constant
LCST	lower critical solution temperature
m	multiplet

MA	methacryloyl
MALDI	matrix assisted laser desorption ionisation
MEHQ	4-methoxyphenol
M_n	number-average molecular mass
M_w	weight-average molecular mass
NCA	N-carboxyanhydrides
NCL	native chemical ligation
NMP	N-methyl pyrrolidone
NMR	nuclear magnetic resonance
MW	microwave
PEG	poly(ethylene glycol)
PDI	polydispersity index
quant.	quantitative
R_f	retardation factor
THF	tetrahydrofuran
TLC	thin layer chromatography

Curriculum Vitae

Maarten van Dijk werd geboren op 3 maart 1980 te Rotterdam. Na het behalen van het VWO-diploma in 1999 werd begonnen met de studie scheikunde aan de universiteit van Utrecht. In het kader van het alfa-gamma project (verplichte studie buiten het eigen vakgebied) behaalde hij in 2002 zijn propedeuse bedrijfskunde aan de Erasmus universiteit te Rotterdam. Na het behalen van het bachelor diploma scheikunde in 2002 begon hij in hetzelfde jaar aan de master Drug Innovation aan de universiteit van Utrecht. In 2002-2003 liep hij stage bij de vakgroep Biofarmacie en Farmaceutische Technologie te Utrecht onder begeleiding van Dr. M.A.E.M. van der AA en Prof. Dr. Ir. W.E. Hennink. Vervolgens liep hij in 2003-2004 stage bij de vakgroep Medicinal Chemistry and Chemical Biology te Utrecht onder begeleiding van Dr. Ir. J.A.W. Kruijtzer en Prof. Dr. R.M.J. Liskamp. In 2004 behaalde hij zijn master diploma, waarna hij in oktober 2004 als aio begon op een gecombineerd project van de vakgroepen Medicinal Chemistry and Chemical Biology en Biofarmacie en Farmaceutische Technologie van het Utrecht Institute for Pharmaceutical Sciences. Onder begeleiding van Prof. Dr. R.M.J. Liskamp en Prof. Dr. Ir. W.E. Hennink werd onderzoek gedaan naar het ontwikkelen van nieuwe methoden voor de synthese van op peptide gebaseerde polymeren. De resultaten van dit onderzoek zijn beschreven in dit proefschrift.

List of Publications

Schiffelers R.M., van Kolfschoten S.C., **van Dijk M.**, Scaria P.V., Woodle M.C., and Storm G. siRNA as a new drug: intellectual property. *Expert Opin. Ther. Patents* **2005**; 15: 141-152.

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Posters

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Title: Synthesis, characterization and enzymatic degradation of peptide-triazole based polymers prepared by microwave-assisted click chemistry
Van Dijk M., Rijkers D.T.S., Liskamp R.M.J, Nostrum C.F., Hennink W.E.

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